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Poly(ethylene glycol)-mesalazine conjugate for colon specific delivery

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ABSTRACT

Chronic inflammatory bowel diseases (IBDs) are still waiting for improved and innovative therapeutic treatments, which can overcome the limits of the current approaches. Since IBDs affect mainly the lower tract of the intestine, a localized therapy in the colon tract will avoid most of the problems caused by systemic or poor selective therapies. Particularly promising are the advance drug delivery systems that can reach specific colon delivery, thus guaranteeing active agent release only at the site of action. This approach can meet two aims at the same time, first of all the drug will not affect healthy tissue and second a lower drug dose may be used because all the administered active agent will reach the target. To obtain a specific colon delivery we exploited the azoreductase enzymes, selectively present only in colon, by inserting an azo linker between a selected drug and a macromolecular carrier. The drug employed is mesalazine, a well know and used agent against IBDs. Poly(ethylene glycol) (PEG), of different molecular weights and structures, was used as carrier. Three different conjugates were synthesized and characterized, and the most promising one, with highest drug loading thanks to the use of diamino PEG of 4 kDa, was further investigated *in vitro* on mouse colonic epithelial cells (CMT-9) and *in vivo* on model mice with induced colitis. The data presented here demonstrate that PEG conjugation of mesalazine prevents drug release and absorption in upper intestine, after oral administration of the conjugates, and that the azo linker ensures a good drug release in the colon tract. The results *in vivo* take into consideration mice bodyweight gain, tissue histology and interleukin-2β as an index of inflammation. These parameters, all together, demonstrated the conjugate effectiveness against the controls.

1. Introduction

Therapeutic treatments for chronic inflammatory bowel diseases (IBDs), such as Ulcerative Colitis (UC) and Crohn's Disease (CD), are still an open chapter. Currently, no curative agents are available and the most promising protocols rely on a long lasting remission that prevents relapses. To achieve this goal, various drugs have been used, like **glucocorticosteroides, immunomodulators and aminosalicylates, and the choice of which drug to employ depends on the activity and distribution of the disease.

Among aminosalicylate derivatives, mesalazine (5-aminosalicylic acid) is the compound of choice for the treatment of IBD. However, this drug lacks specificity and its rapid absorption through the mucosa of upper gastrointestinal (GI) tract hampers a good bio-availability in colonic mucosa and can predispose to the development of a nephrotic syndrome as side effect (Jung et al., 2006).

As a matter of fact, highest efficacy and least toxic effects of an anti-inflammatory therapy occur when the therapeutic agent is specifically delivered to the affected area. For this reason, advance drug delivery systems thoroughly designed to selectively deliver these drugs will be a lot helpful in solving many drawbacks of the currently therapeutic approaches. In the case of IBD, a system that specifically targets the colon will present several and desired advantages in treating the disease localized in that segment of GI tract.

Several approaches have been so far utilized to guarantee colon specific delivery of drugs, such as pH sensitive polymers coatings (Touitou and Rubinstein, 1986; Peeters and Kinget, 1993), time dependent coatings (Saffran et al., 1991; Van den Mooter et al., 1992, 1993; Milojevic et al., 1995; Kalala et al., 1996), biodegradable polymer matrices and hydrogels (Rubinstein et al., 1993; Bronsted et al., 1995) and pro-drugs (Kopecek et al., 1992; Jung et al., 2000; Kopecek, 1990; Sakuma et al., 2001). Colon specific enzymes have been largely studied to trigger selective drug release at this site. Among these, particularly interesting are the azoreductases that catalyze a reductive cleavage of azo linkages. In the study proposed here, these enzymes have been exploited to prepare both low

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molecular and macromolecular pro-drugs of the anti-inflammatory agent mesalazine. The prototype of this class of pro-drugs is sulfasalazine that is converted by the colon azoreductases into active mesalazine and sulfapyridine. Unfortunately, even though this pro-drug allows a local targeting, a lot of side effects and toxicity occur due to the release of sulfapyridine. A new generation of pro-drugs is characterized by a non-toxic (low molecular weight?) carrier linked through a azo bridge to the active drug. The molecules Ipsalazide and Balsalazide are now in the market with the commercial name of Balzide® (Menarini International-L) and Colazal® (Salix Pharm.). Polymeric pro-drugs of mesalazine have also been investigated by linking the drug by means of an azo linkage or an azo containing spacer. For example, as polymeric carrier Kopecek et al. employed *N*-(-hydroxypropyl)methacrylamide (Kopecek et al., 1992; Kopecek, 1990) and Schacht et al. used polyamides (Schacht et al., 1996), both obtaining encouraging results.

This paper reports the preparation and the biological investigation of a polymeric mesalazine pro-drug, obtained by coupling the drug to poly(ethylene glycol) (PEG) through an azo linkage. The cleavage of this azo moiety by the azoreductases, selectively present in the colon, will release mesalazine specifically in that GI tract. The advantages of PEG as polymeric carrier (Greenwald, 2001; Pasut and Veronese, 2007; Duncan, 1992) are the absence of toxicity and immunogenicity, non-absorption along the gastroenteric tract, and last but not least the FDA approval for human use. These properties are making PEG a polymer of choice in drug delivery research for preparation of conjugates with peptide or oligonucleotides drugs, and important products have already reached the market.

2. Materials and methods

2.1. Chemical

Solvents and reagents were from Aldrich Chemical Co. and Carlo Erba Reagenti (Milano, Italy). The PEGs, mPEG-NH_2 (MW 5886 Da), NH_2-PEG-NH_2 (MW 3926 Da) (PEG diamino), mPEG$_2$-COOH (MW 10,870 Da) were from Nektar (Alabama, USA).

^{1}H NMR spectra were recorded on 300 MHz Bruker Biospin spectrometer. Chemical shifts (δ) are reported in parts per million (ppm) downfield from TMS used as internal standard. RP-HPLC analysis were performed with Jasco HPLC system (Japan) equipped with an Jupiter C18 analytical column (5 μm, 250 mm × 4.6 mm) (Phenomenex, USA), eluted with: MilliQ grade water containing 0.05% of TFA (v/v) (eluant A) and acetonitrile containing 0.05% of TFA (v/v) (eluant B), see this section. DEAE 50 ionic exchange resin was purchased from (Pharmacia, Canada).

2.2. Animals

Male Balb/c mice, 10–12 weeks old, were purchased from Charles River (Oderzo, Italy). Mice were housed in groups of four *per* cage and kept under controlled temperature and humidity. Male Wistar rats, weighting 200 g, were also obtained and treated as above. All animals received standard pelleted chow and tap water *ad libitum*, unless otherwise specified. Animal studies were approved by the Institutional Animal Care and Use Committee of the University of Padua.

The homogenization of samples was performed with a Retsch MM300 (QIAGEN, Italy).

The chromatographic system for the quantification of the mesalazine content in the tissues consisted of a separation module 2695 (Waters S.p.A., MI, Italy) and a Coulochem II (Model 5200A) dual potentiostat electrochemical detector equipped with an RS232 interface. Data collection and system control were performed using a PC-based data station (Model 5011A). Separation of analytes was achieved on a reverse-phase DHBA-250 column (5 μm, 250 mm × 3.2 mm). Analytes were detected on a dual electrode analytical cell (Model 5011A, Alfatec S.p.A., Genova, Italy) with the first electrode (E1) set to oxidize the BHBAs at +250 mV and the second electrode (E2) set to oxidize mesalazine at +750 mV. A guard cell was placed between the pump and the autosampler at a potential of +775 mV to oxidize contaminants in the mobile phase. All equipment was from ESA (Chelmsford, MA, USA). The mobile phase consisted of 50 mM sodium acetate, 50 mM sodium citrate, 8% methanol, 2% 2-propanol (v/v). The pH of the mobile phase was adjusted to 2.5 with phosphoric acid and passed through the system at 0.5 ml/min. All analyses were performed at 27 °C. Reference run were performed with solutions containing known amounts of mesalazine (Sigma, MI, Italy).

The mouse colonic epithelial cell-line CMT-93 was obtained from the European Collection of Cell Cultures (ECACC No. 89111413).

2.3. Synthesis of mPEG-PABA-NN-SA (**6**)

2.3.1. mPEG-PABA (**3**)

mPEG-NH_2 (**1**) (1 g, 0.1699 mmol) was added into 50 ml of toluene and dried by azeotropic distillation. After cooling the solution to 0 °C, *p*-aminobenzoic acid, PABA, (**2**) (22.8 mg, 0.1699 mmol) was added. The solution was added of 20 μl of TEA and the temperature was kept at 0 °C. Then HOBT (45.92 mg, 0.3398 mmol) and EDC (67.04 mg, 0.3398 mmol) were added. The reaction was let to proceed under stirring for 24 h. The solvent was removed by evaporation and the oil dissolved in 150 ml of hot ethanol. The product **3** formed a white precipitate after 2 h at 0 °C; this was filtered and dried under vacuum. Yield 909.1 mg (88.8%).

^{1}H NMR, DMSO-d_6. δ (ppm): 3.2 (3H, s, OCH_3, PEG); 3.5 (533H, CH_2 PEG); 5.55 (2H, s, NH_2 PABA); 6.5–7.55 (4H, H arom. PABA); 8.0 (1H, t, NH-CO).

2.3.2. mPEG-PABA-NN-SA (**6**)

mPEG-PABA (**3**) (99.5 mg, 0.0165 mmol) was solubilised in 5 ml of 0.1N HCl at 0 °C. To the mixture 1 ml of 0.25N $NaNO_2$ were added. In another vial 2.65 mg of sodium salicilate (0.0165 mmol) were dissolved in borate buffer pH 9.3. The first solution was dropped into the sodium salicilate solution and the mixture was stirred overnight. Then the pH was brought to 3 using HCl 0.1N, and the product **6** was extracted by CH_2Cl_2 (5× 30 ml). The organic phase, dried over Na_2SO_4, was concentrated in rotavapor. The recovered oil was diluted with 150 ml of hot ethanol. This solution was slowly cooled to room temperature and then kept at 4 °C for 2 h. The product **6** formed a precipitate that was recovered by filtration and dried under vacuum. Yield 80.6 mg (79.18%).

The product **6** was characterized by RP-HPLC, using the conditions reported in Section 2.1 and the following elution gradient: $t = 0'$ $A = 90\%$, $t = 28'$ $A = 20\%$, $t = 30'$ $A = 10\%$, $t = 36'$ $A = 90\%$. Detection was conducted at 360 nm. Elution time for **6** was 17.975′.

^{1}H NMR in DMSO-d_6: δ (ppm): 3.5 (3H, s, –OCH_3, PEG); 3.7 (530H, CH_2 PEG); 7.1 (1H, d, H_d SA); 7.4 (1H, dd, H_c), 6.30–7.1 (4H, arom. PABA); 8.1–8.25 (3H, H arom. SA); 8.5 (1H, d, H_a); 8.8 (1H, t, NH-CO).

2.4. Synthesis of mPEG$_2$-PABA-NN-SA (**7**)

2.4.1. mPEG$_2$-piperazine

Piperazine-BOC (45 mg, 0.2421 mmol) was dissolved in 15 ml of dried chloroform the solution adjusted to pH 8 with TEA and 438 mg (0.04035 mmol) of mPEG$_2$NHS (MW 10,870 Da) were added portion wise. The mixture was stirred at room temperature for 18 h and extracted with 1N HCl (3× 20 ml). The organic phases were dried over Na_2SO_4 and concentrated to small volume. The product,

$mPEG_2$-piperazine-BOC, was diluted with TFA and stirred for 1 h, at the end the solvent was removed by evaporation and the oily residue dropped on diethyl ether to yield 360 mg of crude product.

For a final purification a DEAE 50 ionic exchange column was used. Elution was carried out with a step gradient of water and 0.01 M NaCl to separate the non-hydrolyzed material. Yield: 83%.

2.4.2. $mPEG_2$-PABA

$mPEG_2$-piperazine (270 mg, 0.02503 mmol) was solubilised in 50 ml of toluene and dried by distillation as above reported. To the solution one equivalent of PABA (3.43 mg, 0.02503 mmol) and 20 μl of TEA, the solution cooled to 0 °C, HOBT (6.76 mg, 0.05 mmol) and EDC (14.38 mg, 0.075 mmol) were added and the reaction mixture that was let stirring for 24 h. The solvent was removed by evaporation and the remaining oil diluted with 150 ml of hot ethanol. After 2 h at 4 °C the product *$mPEG_2$-PABA* formed a white precipitate that was filtered and dried under vacuum. Yield 217.50 mg (79.15%).

^{1}H NMR, DMSO-d_6. δ (ppm): 1.3 (8H, CH_2 Lys; 3.1 (6H, $-OCH_3$ PEG); 3.3–3.5 (CH_2 PEG); 3.8 (1H, dt); 5.3 (2H, s, NH_2 PABA); 6.35–6.9 (4H, arom. PABA); 7.0 (1H, t); 7.25 (1H, d).

2.4.3. $mPEG_2$-PABA-NN-SA (**7**)

$mPEG_2$-PABA (100 mg, 0.0091562 mmol) was solubilised in 2 ml of 0.1N HCl at 0 °C and 0.5 ml of a 0.25N $NaNO_2$ solution was added drop wise. A second solution was prepared dissolving 1.26 mg (0.0091 mmol) of sodium salicilate in borate buffer pH 9.3. The first solution was added drop wise to the second one and left under stirring overnight. The mixture was brought to pH 3 with HCl 0.1N and extracted with CH_2Cl_2 (3× 30 ml). The organic phase, dried over Na_2SO_4, was reduced to few milliliters and diluted with 150 ml of hot ethanol. After cooling at 4 °C an orange precipitate was recovered by filtration and dried under vacuum. Yield 74 mg (73%). The product (**7**) was analyzed by RP-HPLC, using the conditions reported in Section 2.1 and the following elution gradient: $t = 0'$ $A = 90\%$, $t = 28'$ $A = 20\%$, $t = 30'$ $A = 10\%$, $t = 36'$ $A = 90\%$.

^{1}H NMR on DMSO-d_6: δ (ppm): 1.2 (8H, CH_2 Lys); 3.0 (6H, $-OCH_3$ PEG); 3.0–3.5 (CH_2 PEG); 3.7 (1H, dt); 6.30–7 (4H, arom. PABA); 7.0–7.9 (3H, arom. SA).

2.5. Synthesis of PEG-(PABA-NN-SA)$_2$ (**8**)

2.5.1. PEG-(PABA)$_2$

PEG-$(NH_2)_2$ (MW 3926 Da), (0.6 g, 0.3538 mmol) was solubilised in 50 ml of toluene and dried by distillation. To the solution PABA (53.37 mg, 0.3893 mmol) was added. 20 μl of TEA were added and the temperature cooled to 0 °C. HOBT (231.38 mg, 0.7786 mmol) and EDC (105.2 mg, 0.7786 mmol) were added in sequence to the solution and the reaction was left to react for 24 h at room temperature. The solvent was removed by evaporation and the product solubilised in 150 ml of hot ethanol. After cooling a white precipitate was obtained and recovered by filtration. Yield 544 mg (83.8%).

^{1}H NMR, DMSO-d_6. δ (ppm): 3.4–3.6 (CH_2 PEG); 5.6 (4H, s, $-NH_2$); 6.55–7.55 (8H, arom. PABA); 8 (2H, t, –NHCO–).

2.5.2. PEG-(PABA-NN-SA)$_2$ (**8**)

The product was synthesized and purified as reported for **7** starting from 544 mg of PEG-$(PABA)_2$.

Yield 80.6 mg (79.18%). The final product (**8**) was characterized by RP-HPLC, using the conditions reported for **7**.

^{1}H NMR, DMSO-d_6. δ (ppm): 3.3–3.6 (CH_2 PEG); 7.75 (2H, d); 6.3–8 (14H, arom. PABA and SA); 7.95 (2H, dd); 8.25 (2H, d).

2.6. Conjugates stability in artificial gastric juice

The artificial gastric juice, prepared according to the FUI XI procedure, consisted of a solution containing 5 mg of NaCl, 8 mg of pepsin, 2 ml of 0.1N HCl and 0.5 ml of water. For each conjugate a solution was prepared by dissolving in 1 ml of artificial gastric juice an amount of conjugate containing 0.17 mg of drug. The solutions were maintained at 37 °C, at predetermined time points a sample of 20 μl was collected and analyzed by RP-HPLC to evaluate the conjugate stability.

2.7. Conjugates stability in artificial pancreatic juice at pH 6.8

The pancreatic environment was simulated by dissolving 3.5 mg of porcine pancreatin in 1 ml of PBS, pH 6.8. Each conjugate, 0.17 mg (drug equivalent), was dissolved in 1 ml of pancreatic solution, and incubated at 37 °C while at scheduled times samples were analyzed by RP-HPLC to evaluate the product stability.

2.8. Conjugates stability in the colonic lumenal content

Male Wistar rats or balb/c mice were sacrificed, the cecum isolated and surgically removed. The luminal content was collected and added to 2 ml of nitrogen bubbled PBS, pH 6.8, whereas the internal mucosa were washed with the same buffer and then removed by scraping with glass. The slurry was then subjected to sonication for 15 min on ice. The conjugates (0.15 mg drug equivalent) were dissolved in 1 ml of nitrogen bubbled PBS, pH 6.8, added to colonic suspension and the final mixture incubated in anaerobic conditions at 37 °C up to 24 h. Samples were taken at selected time intervals, centrifuged and the supernatant analyzed by RP HPLC to evaluate the mesalazine (**9**) release.

2.9. Behavior of conjugates in vivo

The conjugate hydrolysis and absorption in the different gastrointestinal tracts was evaluated in mice ($n = 3$ *per* group) randomly allocated to receive either vehicle, mesalazine conjugate or mesalazine (2 mg/kg body weight, expressed as free drug). After 2 and 6 h the animals were killed and a blood sample collected in heparin coated tubes, centrifuged and the plasma stored at −80 °C. The intestine was rapidly removed, the ileal and colonic mucosa collected and a fecal sample by the descending colon was collected also. Tissue and fecal samples, stored at −80 °C until used, were homogenized in 0.2 M PCA, perchloric acid, (containing 100 μM sodium metabisulfite) 1:10 (w/v) at 4 °C. Samples of 10 μl were centrifuged (13,000 × *g*, 10 min, 4 °C). The clear supernatant filtered through a 0.2 μm filter, was analyzed with a chromatographic system equipped with an electrochemical detector.

2.10. Induction of colitis by dextran sodium sulfate (DSS) and assessment of colitis

To induce colitis balb/c mice received drinking water supplemented with 4% (wt./vol.) dextran sodium sulfate DSS (TDB Consultancy, Uppsala, Sweden) for 7 days. Experimental animals were randomly divided into groups receiving daily either PBS (control group), azo PEG conjugate (**8**) (5 mg/kg body weight) or mesalazine (2 mg/kg body weight) by gastric gavage. To assess colitis severity a previously validated clinical score ranging from 0 to 4 was calculated using the parameters of weight loss, stool consistency, and the presence or absence of fecal blood (Brun et al., 2005). Mice were killed, the colon was removed and a segment from the proximal colon were fixed in 4% PFA, paraffin embedded and then

longitudinal sections (10 μm thick) stained with haematoxylin and eosin (H & E) were subjected to histological evaluation using a scoring system previously validated and described. The system takes into account three independent parameters: severity of inflammation, depth of injury and crypt damage (Castagliuolo et al., 2005). In addition two other full thickness samples from the proximal colon were snap frozen in liquid nitrogen to quantify myeloperoxidase (MPO) activity and IL-1β levels.

2.11. Determination of colonic IL-1β levels

A full thickness fragment of the proximal colon was homogenized in ice-cold phosphate buffer saline (pH 7.4) containing a mixture of protease inhibitors (1 μM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin). Homogenates were centrifuged at 20,000 × *g* for 10 min at 4 °C and supernatants collected and stored at −80 °C until IL-1β levels were measured by a commercially available ELISA kit (Endogen, Woburn, MA). IL-1β levels were expressed as pg per mg total proteins.

2.12. Measurement of MPO activity

To determine colonic myeloperoxidase levels, an index of neutrophils infiltration, full thickness segments of the proximal colon were weighted and homogenized in 50 mM KH_2PO_4 buffer (pH6) containing 0.5% hexadecyltrimethylammonium bromide (ratio 1:10, w/v). Homogenates were then centrifuged 10,000 × *g* (10 min at 4 °C), the clear supernatants collected and MPO activity measured using a colorimetric assay method and expressed as U per mg of tissue.

2.13. Mesalazine quantification by HPLC in biological samples

We quantified mesalazine in the ileal and colonic mucosa and in the plasma following oral administration, using a modification of the method by McCabe et al. (1997).

2.14. Cell culture and in vitro cytotoxicity assay

A mouse colonic epithelial cell line, CMT-93 cells, was maintained in Dulbecco's modified Eagle medium (GIBCO) supplemented with 10% heat inactivated fetal calf serum (GIBCO), 100 U/ml penicillin and 100 μg/ml streptomycin (complete medium). Sub confluent monolayers were trypsinized, and then seeded at a density of 2×10^4/cells in 96-wells plates and grown for 24 h in complete medium. The cytotoxic activity of different compounds was determined using a standard 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazodium bromide (MTT)-based colorimetric assay (Ferlin et al., 2005). After 24 h, exponentially growing cells were washed twice with warm sterile DMEM and incubated with 100 μl with fresh medium alone (control) or containing PEG-conjugates (0.01–100 μM) or mesalazine (0.01–10 μM) for 24–72 h. Then cell survival was assessed by addition of a MTT solution (10 μl of 5 mg/ml MTT in phosphate saline buffer). After 4 h were added 100 μl of 10% SDS in 0.01N HCl, and the plates were incubated at 37 °C for a further 18 h. Then optical absorbance was measured at 550 nm using a LX300 Epson Diagnostic microplate reader. Survival ratios were expressed in percentage values with respect to untreated cells and determined from replicates of 6–8 wells from at least two independent experiments.

2.15. Statistical analysis

Results are expressed as mean ± standard error. Statistical analysis was performed using ANOVA and Bonferroni's test. Statistical significance was considered for p values < 0.05.

3. Results and discussion

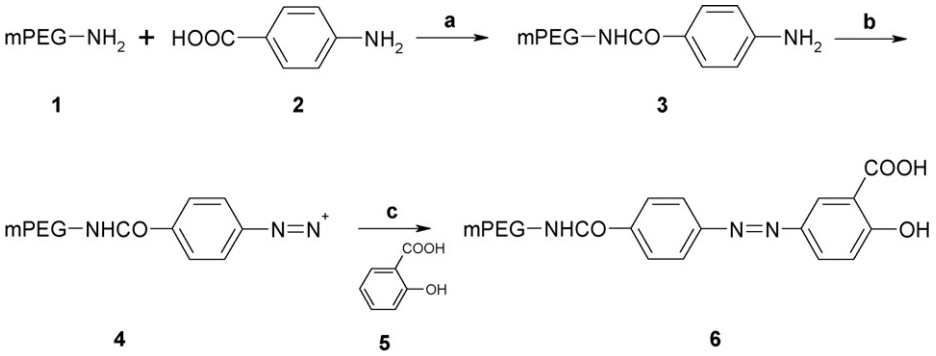
3.1. Conjugates synthesis and characterization

Three mesalazine PEG-conjugates were synthesized, according to Schemes 1 and 2. The polymer coupling was performed with the aim to prevent the direct absorption of the drug in upper gastrointestinal tract. In fact, PEG, at the molecular weight used in this study, cannot be absorbed through the intestine (Ryan et al., 1992). To achieve specific colon release of the conjugated drug an azo linkage between PEG and drug was chosen. PEGs differing in molecular weight and structure were employed, in particular mono-functional (linear or branched) or di-functional (diamino) PEGs. The last has the advantage of higher drug loading while the branched one can better prevent the conjugate absorption along the GI tract. On the other hand, this branched PEG presents a lower drug payload and tends to form solutions with higher viscosity than the low molecular weigh PEGs.

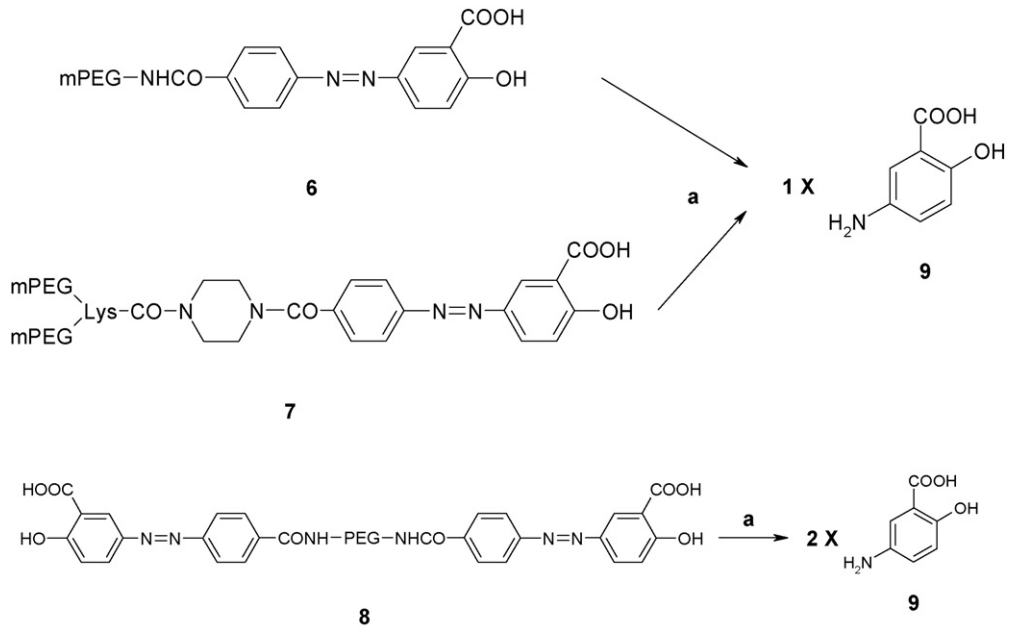
The three conjugates (PEG-(PABA-NN-SA)$_2$, mPEG$_2$-PABA-NN-SA, mPEG-PABA-NN-SA) were obtained in high yield and the identity and purity were assessed by RP-HPLC analyses and ^{1}H NMR spectroscopy. The chemical shifts for each compound and intermediate are reported in Section 2.

3.2. Conjugates stability in artificial gastric and pancreatic juices

As expected from previous studies on different polymer-drug azo conjugated (Sinha and Kumria, 2001), also these PEG mesalazine azo conjugates were stable in gastrointestinal environment. This investigation was carried out by incubating the



Scheme 1. Synthesis of mPEG-PABA-NN-SA (**6**); a: EDC, HOBt, TEA, CH_2Cl_2; b: $NaNO_2$, HCl, 0 °C; c: borate buffer 0.2 M, pH 9.



Scheme 2. Structure of the three PEG-drug conjugate and release of mesalazine (**9**) following azoreductase incubation: linear monoazo, mPEG-PABA-NN-SA (**6**), biazo, PEG-(PABA-NN-SA)$_2$ (**8**), branched mPEG$_2$-PABA-NN-SA (**7**).

conjugates in gastric and pancreatic artificial juices and evaluating the drug release by RP-HPLC analysis. The amide bond between PEG and PABA (see Scheme 1 for example) was also stable against the proteolytic enzymes present in the gastrointestinal tract, this may be likely due to both the steric hindrance of PEG polymer chain and PABA aromatic ring.

3.3. Mesalazine release by incubation with the colonic lumenal content

Mesalazine was released from each PEG-conjugate after incubation with a lysate of mice colonic flora (Fig. 1). The release pattern from PEG-(PABA-NN-SA)$_2$ (**8**) (azo PEG) was biphasic, most likely because it takes place at different rates from the di-substituted and the mono-substituted species. We speculate that the last, formed after the release of the first drug molecule from the di-substituted **8**, may form micelles that protect better the azo linkage, as demonstrated for other PEG-drugs (Veronese et al., 2005; Visentin et al., 2004).

3.4. Colonic absorption of mesalazine following oral administration

Fig. 2 reports the mesalazine amount detected in ileum and colon mucosa at 2 and 6 h after oral administration of the free drug or the conjugate obtained from the diamino PEG, PEG-(PABA-NN-SA)$_2$ (**8**). Mesalazine was not detectable in ileal mucosa while it was present in animals receiving free mesalazine. On the other hand the released free drug was found in colon mucosa where the concentration was significantly higher than that found in animals receiving mesalazine.

These results demonstrated that the higher hindrance of the branched conjugate, is not needed to prevent intestinal absorption and therefore it was not considered for the following animal experimentation, furthermore among the two linear conjugates **6** and **8**, only the second, obtained with the diamino PEG, namely PEG-(PABA-NN-SA)$_2$ was used in the following biological experimentations, thanks to its higher loading.

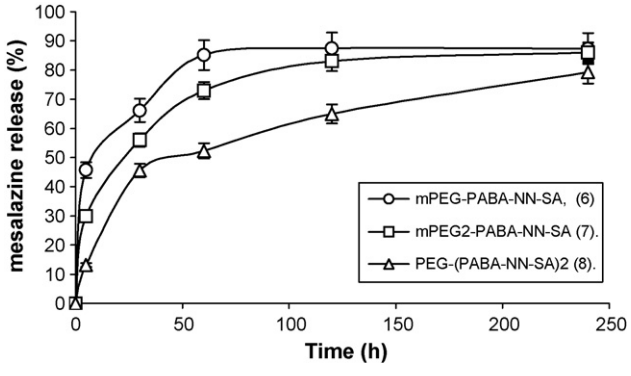


Fig. 1. Time course of mesalazine release from conjugates by azoreductase reduction of azo linkage, as evaluated by RP-HPLC: mPEG-PABA-NN-SA **6** (○), mPEG2-PABA-NN-SA **7** (□) and PEG-(PABA-NN-SA)$_2$ **8** (△).

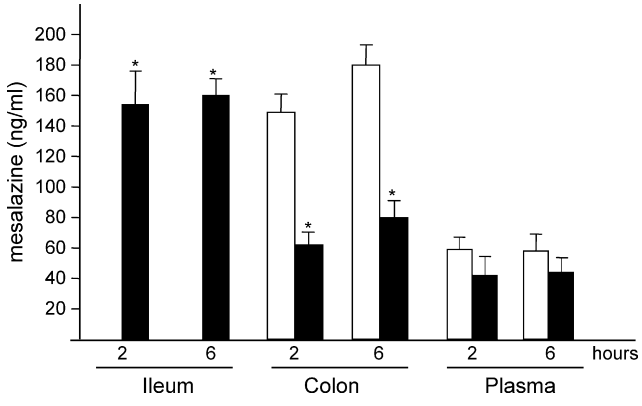


Fig. 2. Mesalazine concentration in ileum, colon and plasma at 2 and 6 h following oral administration of free (white column) or conjugated drugs (black column) as evaluated by dual potentiostatic electrochemical detector.

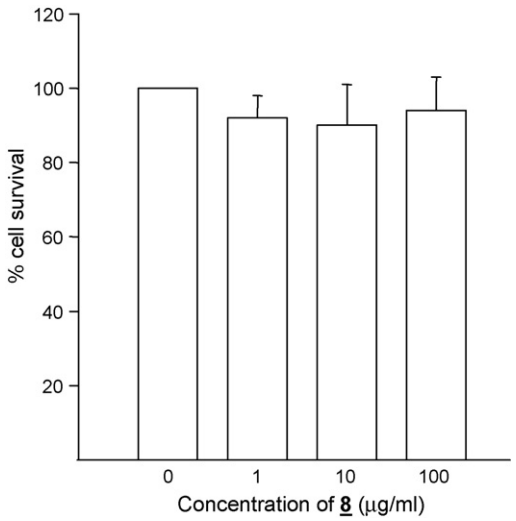


Fig. 3. Survival of colon epithelial cell line, CMT-93 in the presence of increasing concentration of PEG-(PABA-NN-SA)$_2$ (**8**).

Table 1
Effect of PEG-(PABA-NN-SA)$_2$ (**8**) on DSS colitis outcome in mice.

	Body weight change (%)	Clinical score	Histologic score
Control	(+) 4.8 ± 0.2	0	0.1 ± 0.1
DSS	(−) 25.1 ± 0.8**	3.3 ± 0.3**	1.9 ± 0.4**
DSS + **8**	(−) 11.8 ± 0.5$^{**,+}$	1.5 ± 0.5$^{**,+}$	1.1 ± 0.2$^{**,+}$
DSS + 5ASA	(−) 10.1 ± 0.6$^{**,+}$	1.2 ± 0.2$^{**,+}$	0.9 ± 0.23$^{**,+}$

Data represent the means ± S.E. of 6–10 animals per group.
** $p < 0.01$ vs. control.
$^{+}$ $p < 0.05$ vs. DSS alone.

Fig. 2 shows also that mesalazine was present in close concentration in plasma after oral administration as free form or conjugated form. The lower concentration observed in plasma for the conjugate may be due to the slow release from the pro-drug form. All of these data are demonstrating that the PEG prevents intestinal absorption of the drug that instead occurs only after the cleavage of the azo-linkage by colonic azoreductase.

3.5. PEG-(PABA-NN-SA)$_2$ toxicity effects on intestinal epithelial cells

Since, by oral administration, the conjugates reach unmodified the colon, where they are degraded by bacterial enzymes to release active mesalazine, we though it useful to investigate the effects of the pro-drug on mouse colonic epithelial cells, CMT-93. Fig. 3 demonstrates that no significant toxic effects could be observed up to 0.1 mg/ml of the azo PEG conjugated.

3.6. Effect of free and PEG conjugated drug on DSS induced colitis

Dextran sodium sulfate, as an agent to induce experimental colitis (Rumi et al., 2004), was administered to mice at a concentration of 4% in the drinking water. Within 4–5 days the animals started to develop signs of colitis such body-weight loss, diarrhea and blood into the feces. Within the 7 days of DSS administration mice lost about 35% of their body-weight, showed a bloody diarrhea and the histological analysis demonstrated the presence of superficial mucosal ulcers associated with an inflammatory infiltrate rich in neutrophils (Table 1 and Fig. 4). Furthermore, myeloperoxidase MPO and IL-1β levels were significantly increased over control animals. As shown in Table 1 and Fig. 4, daily intragastric administration of PEG conjugate significantly reduced DSS-induced inflammatory damage, as shown by reduced body weight loss and

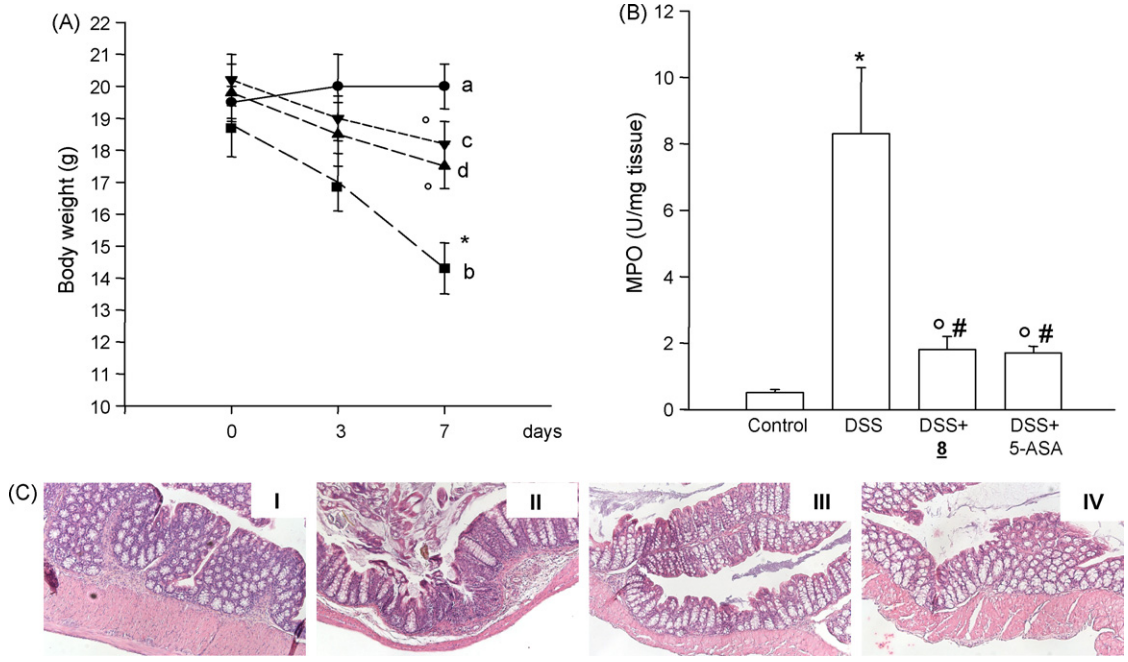


Fig. 4. Protective effects of treatment with PEG-(PABA-NN-SA)$_2$ (**8**) (c) and free mesalazine (d), during DSS colitis (b) with respect to control (a). Data are expressed as means ± S.E.M. $^{*}p < 0.01$ vs. control; $^{\#}$<0.05 vs. control; $^{\circ}p < 0.01$ vs. DSS alone. Severity of colitis was monitored by measure of body weight (panel A). At the end of the experiment animals were killed, and full-thickness samples of the proximal colon, collected to determine MPO activity that expressed as U/mg wet tissue (panel B), or fixed in 10% PFA, paraffin embedded and sections stained with H & E were analyzed by a pathologist in a blinded fashion. Panel c (original magnification 10×) shows a representative colonic section from a control mouse (I), a mouse receiving DSS alone (II), treated with conjugate **8** (III) or mesalazine (IV). Mucosal ulceration and inflammatory cell are evident in DSS mice receiving only vehicle. The inflammatory infiltrate is drastically reduced and the mucosal structure is conserved in conjugate **8** and mesalazine treated mice (compare II with III and IV). Results are expressed as means ± S.E.M. $^{*}p < 0.01$ vs. control; $^{\#}$<0.05 vs. control; $^{\circ}p < 0.01$ vs. DSS alone (panel C).

the blunted increase in the colonic mucosa of MPO activity and IL-1β level. As expected, also the severity of the histologic damage was drastically reduced in animals receiving the conjugate daily.

4. Conclusions

Therapy based on administration of polymeric pro-drugs is an approach that receives a continuously increasing interest (Pasut and Veronese, 2007), because the polymer can convey special advantages to drugs, such as (i) *in vitro* and *in vivo* increased stability, (ii) tumour and inflamed tissue localization, by the known EPR effect (Maeda and Matsumura, 1989), (iii) increased body residence time and (iv) reduction of antigenicity.

For specific colon release azo-linkages may be exploited because these chemical moieties are stable in the upper gastrointestinal tract (Scheline, 1973), providing that pro-drugs reach unmodified the colon, where the drug can be promptly released by the action of azoreductases enzymes that are present only in the last tract of GI. It is noteworthy that the reductive cleavage acted by these enzymes lead to the formation of the corresponding amino-drug (Schacht et al., 1996; Schroder and Jhoansson, 1973; Miyadera, 1975).

The data reported in this paper demonstrate that PEG conjugation of mesalazine prevents drug release and absorption in upper intestine, after oral administration of the conjugates, and this also without the need of the high mass and sterically hindered branched PEG, as present in the compound **7**. The drug absorption in the conjugated form is prevented because the intestinal permeability of PEG is negligible for molecular weight above 3000 Da. Conjugate **8** was further and thoroughly tested, in model mice with colitis and in mouse colonic epithelial cells (CMT-9), because its higher drug loading with respect to compound **7** makes this conjugate a lead and suitable candidate for future developments. This product was easily prepared with high yield starting from salicylic acid (SA), thanks to the fact that the SA hydroxyl group directs the diazotization in the *para* position, thus allowing the insertion of an azo linkage in the same site of the amino group of mesalazine (Wiwattanapatapee et al., 2003). Therefore the reduction of this azo group, by colon azoreductases, leads to formation and release of mesalazine.

The therapeutic activity of the conjugate **8**, assessed in mice with induced colitis, was very encouraging. Mice bodyweight gain, interleukin-2β as an index of inflammation and tissue histology were all together evaluated to prove the conjugate effectiveness against the controls.

In addition, the results obtained here with this polymeric pro-drug, might open the way for a more general application of PEG for specific colon delivery, providing that a suitable azoreductase cleavable linker can be used for drug conjugation.

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