Full Paper

Potential Tuberculostatic Agent: Micelle-forming Pyrazinamide Prodrug

Marcia Silva[1], Nara L. Ricelli[2], Omar El Seoud[3], Celso S. Valentim[4], Antônio G. Ferreira[5], Daisy N. Sato[6], Clarice Q. F. Leite[1], Elizabeth I. Ferreira[2]

[1] Faculdade de Ciências Farmacêuticas, Universidade Estadual Paulista, Araraquara, Brazil
[2] Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, São Paulo, Brazil
[3] Instituto de Química, Universidade de São Paulo, São Paulo, Brazil
[4] Instituto de Química, Universidade Estadual Paulista, São Paulo, Brazil
[5] Centro de Ciências Exatas e de Tecnologia, Universidade Federal de São Carlos, São Paulo, Brazil
[6] Instituto Adolfo Lutz, Ribeirão Preto, São Paulo, Brazil

Pyrazinamide was condensed with the poly(ethylene glycol)-poly(aspartic acid) copolymer (PEG-PASP), a micelle-forming derivative was obtained that was characterized in terms of its critical micelle concentration (CMC) and micelle diameter. The CMC was found by observing the solubility of Sudan III in Poly(ethylene glycol)-poly(pyrazinamidomethyl aspartate) copolymer (PEG-PASP-PZA) solutions. The mean diameter of PEG-PASP-PZA micelles, obtained by analyzing the dynamic light-scattering data, was 78.2 nm. The PEG-PASP-PZA derivative, when assayed for anti-*Mycobacterium* activity, exhibited stronger activity than the simple drug.

Keywords: Pyrazinamide / Micelle-forming polymer / Tuberculostatic prodrug

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Introduction

The impact of tuberculosis (TB) on the morbidity and mortality rates in different parts of the world has varied widely throughout human history and, while it arises from a bacterial infection, the prevalence of this disease in a community is strongly influenced by socioeconomic factors. Abuse of drugs, tobacco and alcohol, crowded conditions, poor hygiene, and malnutrition favor the spread of *Mycobacterium tuberculosis*, enabling TB to add to the number of its victims, chiefly in less well-off sectors of the population. However, the disease also attacks members of other classes, especially when their immune system is suppressed for some reason, a situation that brings everyone to the same level. The current extent and seriousness of the advance of TB may be expressed simply in terms of the burden it puts on society, undermining its very structure; thus it kills more women than all other causes of death put together, kills more young people and adults than any other infectious disease, and probably leaves more orphans than any other infection. TB is responsible for 32% of HIV-positive patient deaths, this being three times more than any other pathological cause (malignant neoplasms: 6%, septicemia 11%, and other infections 10%) [1, 2].

The route of infection is by exhalation and inhalation of *M. tuberculosis* bacteria, which grows in protective tubercles in the lungs, making it hard to protect the host and those nearby. Hence, an infected patient must be detected early and given uninterrupted treatment thereafter, so as to try and reduce the risk of infecting the community. Another problem is that the bacterium may stay in a person for life, ready to be activated at any time, and is capable of exacerbating other diseases or substituting them as the cause of death. At first, TB may be confused with other respiratory diseases, and the consequent delay in reaching a correct diagnosis gives time for the bacteria to be disseminated and for the state of the patient to wor-

Correspondence: Marcia Silva, Faculdade de Ciências Farmacêuticas, Universidade Estadual Paulista – Fármacos e Medicamentos, CP502, Campus Araraquara Sao Paulo 14801-210, Araraquara – SP, Brazil.
E-mail: silvam@fcfar.unesp.br
Fax: +55 16 3301-6960

sen. In many cases, by the time the infection has been diagnosed as *M. tuberculosis*, the host has already passed it to others, at a rate of 12–15 people per annum. In epidemiological terms, TB now occupies a prominent position among causes of death, especially in young adults (15–49 years), and there is considerable concern over its potential impact on socioeconomic development. The number of people at risk of developing TB is alarming: it is estimated that around 30% of the world population, 1.7 billion people, are carrying *M. tuberculosis* and that one person is infected every second [1, 2].

The impact of tuberculosis worldwide demands new approaches, on both the clinical and socioeconomic fronts so that effective therapy is provided at the point of need and appropriate social action taken [3, 4]. It is certainly important to improve our understanding of the pathogenesis of TB, the immune response to it, and its mechanisms of resistance by studying the genetics and biology of *M. tuberculosis* in depth. However, it cannot be denied that the discovery and development of new drugs, hand-in-hand with better diagnostic methods, social advances in the most-affected sectors and public health programs to combat TB could bring us within reach of the long sought-after goal of defeating this disease.

In this context, the present study was intended to contribute to the development of new anti-TB drugs, in particular a polymeric prodrug derived from a poly(ethylene glycol)-poly (aspartic acid) block copolymer by substituting pyrazinamide on the aspartate free carboxylic groups (The reactions involved in the synthesis of the polymeric prodrug derived from pyrazinamide are outlined in Scheme 1). The substituted polymer forms a micelle, with a hydrophobic central region consisting of the drug-ligand group and a hydrophilic outer coat [5, 6]. The advantage of this micellar transporter over others lies in the ability to control the particle size, its structural stability, and good solubility in water. In addition, this structure allows a controlled rate of delivery of the drug, reduced toxicity and selective action on the chosen target [6, 7]. The potential for prolonged drug action at low toxicity level means that this system could lead to greater patient approval, which in turn would discourage abandonment of the treatment, which is one of the most common causes of failure and the spread of resistance. Furthermore, when the size of the micelle is suitably adjusted, it can be absorbed by the alveoli, efficiently targeting the primary site of infection.

The development of micelle-forming polymeric prodrugs from poly(ethylene glycol)-poly (aspartic acid) copolymer derivatized with pyrazinamide takes advantage of the valuable properties of the components. Poly(ethylene glycol) has been used to modify the surface interactions of proteins, reducing their antigenicity, and of microspheres, reducing their rate of capture by the liver. This polymer is neither toxic nor immunogenic, but is soluble in water, and thus, as a constituent of the outer coat of the micelle, has the property of inhibiting interactions with biocomponents: proteins and cells [8–10, 13]. Poly(aspartic acid) forms the hydrophobic segment, together with the tuberculostatic ligands. This synthetic polyaminoacid has carboxylic groups capable of forming biodegradable ester or amide bonds. Besides, this polymeric chain, when hydrolyzed, generates aspartic acid, an amino acid that takes part in biochemical processes both of the host and the bacterium.

The treatment of TB requires several drugs to be taken together to increase their effectiveness by synergy and, especially, to overcome resistance of the microorganism [1–4]. Although many drugs exist that can be used in courses of treatment for TB, few of these would be described as drugs of choice, when evaluated in terms of effectiveness and toxicity. These few include pyrazinamide, isoniazid, and rifampicin. Pyrazinamide is a structural analogue of nicotinamide, used as a chemotherapeutic agent together with other tuberculostatic drugs, against all types of TB. Its activity is pH-dependent, and is limited to slow-growing bacilli. Owing to its toxic effect on the liver, it has to be used under close medical supervision, accompanied by regular tests of liver function.

In light of the above, the possibility of developing systems (prodrugs) that release the drug during treatment, achieving equal or greater effectiveness, lower toxicity and prolonged action, raises the hope of eventually controlling TB, albeit in the long term. It should be stressed that the prolonged action of prodrugs should lead to improved patient adhesion to their treatment regimens, which in turn would diminish the rate of appearance of pathogens resistant to the corresponding drugs.

Results and discussion

Critical micelle concentration

The drug derivative PEG-PASP-PZA is an amphiphilic polymer capable of forming micelles. To evaluate this process, the critical micelle concentration (CMC) was estimated by plotting the solubility of Sudan III in polymer solutions of varying concentrations. From the sharp bend in the absorbance curve in Fig. 1, the CMC of PEG-PASP-PZA was determined as 5.0×10^{-3} mg/L.

Micelle diameter

Diameters of the micelles formed in aqueous solution (1% w/v) were estimated by dynamic light-scattering

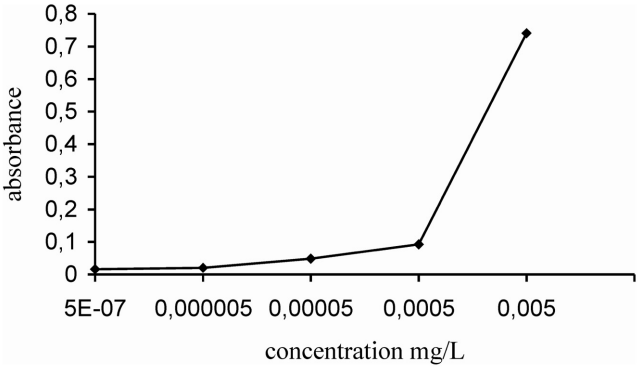


Figure 1. Absorbance curve for critical micelle concentration (CMC).

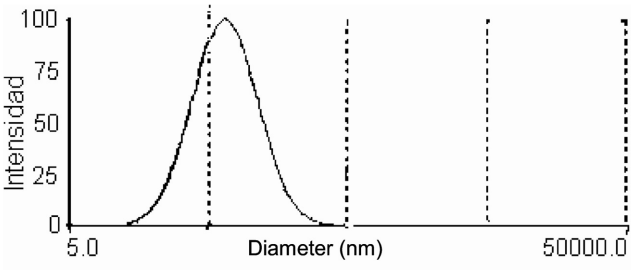


Figure 2. Distribution of micelle sizes.

(DLS). The distribution of micelle sizes is shown in Fig. 2, the median diameter of PEG-PASP-PZA micelles being 78.2 nm.

Biological assay

The MICs of PEG-PASP-PZA and precursors, against strains Ra and Rv of *M. tuberculosis*, were estimated by observing the color change produced by growing bacteria in Alamar Blue, in the presence of a range of dilutions of these compounds, in the wells of a culture microplate. Stock solutions of 8.9 mg/mL were prepared, and dilutions from 89.0 to 5.56 μg/mL were tested for inhibition of growth of the two strains, in culture media of pH 7. In a preliminary test, hydroxymethylpyrazinamide was assayed against strain Ra and showed a MIC of 24.8 μg/mL. The polymeric derivative of PZA also exhibited antibacterial activity; the results are displayed in Table 1.

The synthesis of BLA (β-benzyl-L-aspartate), NCA-BLA (β-benzyl-L-aspartate-*N*-carboxyanhydride), PEG-PBLA (Poly(-ethylene glycol)-poly(β-benzyl-L-aspartate)), and PEG-PASP was successful, likely was reported elsewhere [14]. The synthetic route of the polymeric drug is show in Scheme 1.

In preliminary experiments, the *N*-hydroxymethylation of PZA was carried out by the method described by

Table 1. Minimum inhibitory concentrations (MIC) on *M. tuberculosis* strains.

Strains	PZA (μg/mL)	PZA-CH_2OH (μg/mL)	PEG-PASP (μg/mL)	PEG-PASP-PZA (μg/mL)	PZA Content (μg/mL)
Ra	≤6.25	24.8	NI[a)]	≤6.25	≤0.475
Rv	12.5	24.8	NI[a)]	12.5	≤0.950

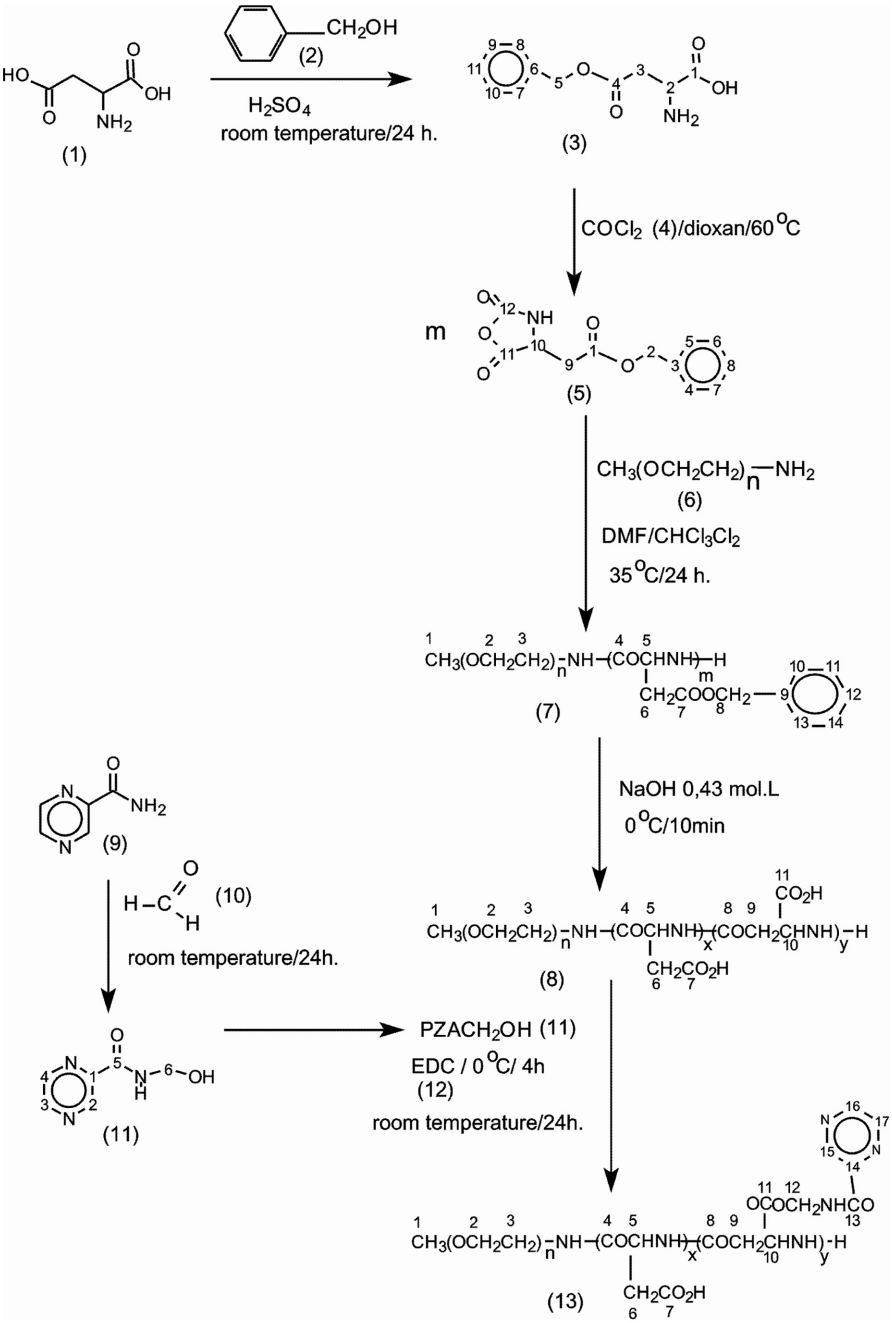
[a)] NI – inhibition factor

Bonina *et al.* [15], but the yield was low (28 – 0%). To overcome this problem, the method was modified slightly: after 20 h of reaction, a further 50% of the formaldehyde was added, and the yield rose to 80%. The measured melting range, 126 – 28°C, was lower than that of the underived PZA, and the derivative was more soluble in water. This occurred because the substitution of $-CH_2OH$ on the amide-N reduced the strength of H-bonding in the crystal, allowing the structure to be more readily disorganized, either by melting or by solvation in water.

The 'spacer' *N*-hydroxymethyl was introduced for two reasons: first, it should modify the reactivity of the PZA, facilitating its condensation with the polymeric carrier, and second it should leave the polymer-bound PZA more accessible to the action of hydrolytic enzymes that liberate the drug at the site of delivery. The ^{1}H- and ^{13}C-NMR spectra show the formation of the product, exhibiting the chemical shifts (δ) 4.54 and 63.40 ppm related to, respectively, the hydrogens and carbon of the methylene group of PZACH_2OH. Hydrogens of the heteroaromatic ring gave rise to shifts at (δ) 8.94 and 8.22 – 8.50. Chemical shifts related to the carbonyl, heteroaromatic, and methylene carbons were identified at 163.93, 148.53, 148.24, 144.53, 144.46, and 62.40 ppm, respectively.

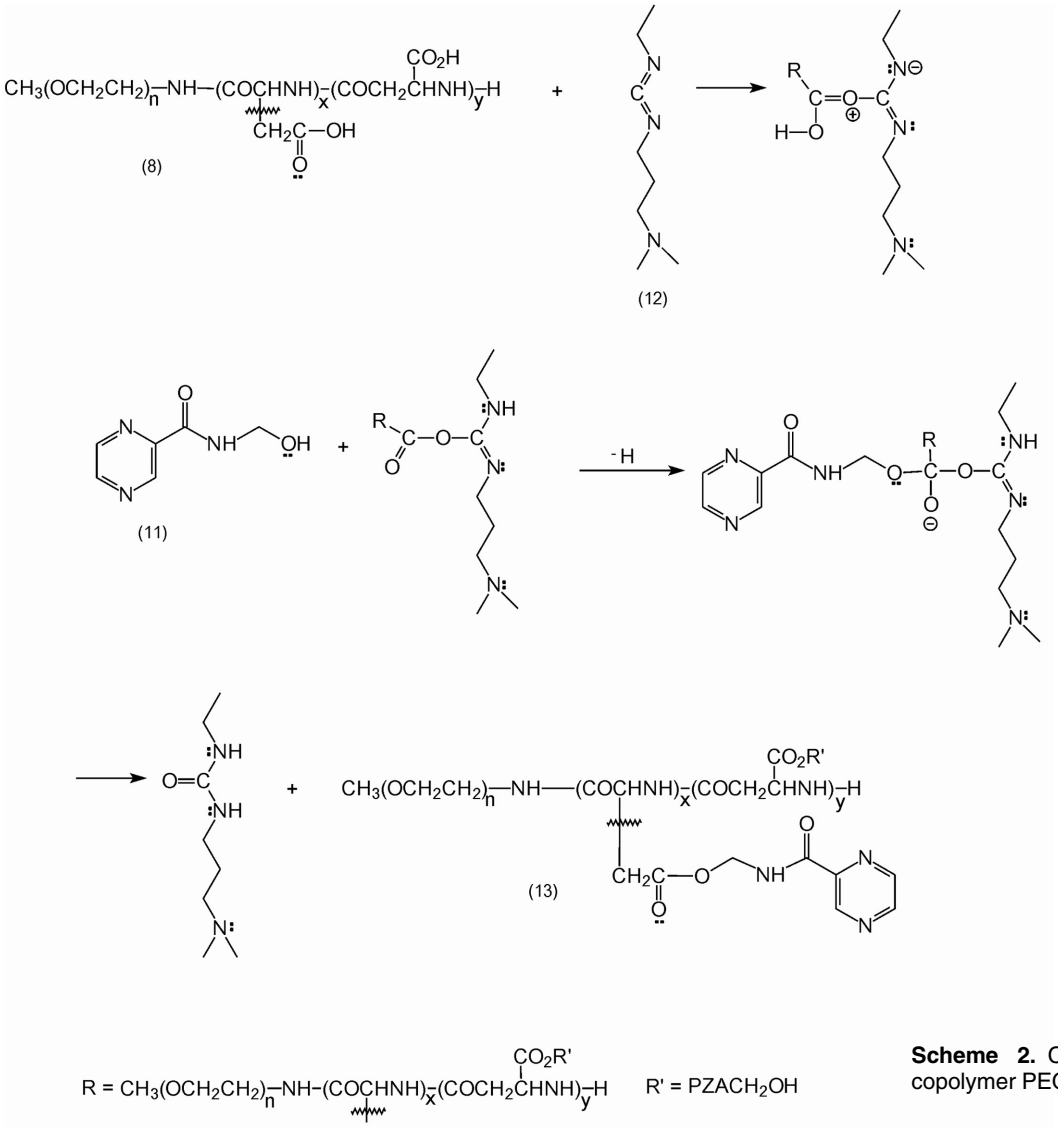
Hydroxymethyl derivatives of acids, in this case an amide, are rather unstable, reverting to the underived compound in the presence of bases. Thus, long after its synthesis, the hydroxymethylpyrazinamide showed a melting range quite different from that observed in the fresh sample; its instability was confirmed by the NMR spectra, which no longer showed the chemical shifts characteristic of the *N*-hydroxymethylated compound. It is possible that this degradation was due, at least partly, to reaction with the storage vessel (probably a basic glass) or with some residual impurity. Following this observation, later samples of PZACH_2OH were recrystallized twice and stored in porcelain devices. With those procedures we verified that the instability is dependent on a proper storage, needing therefore to be stored in a porcelain vessel.

The approach used in the condensation of copolymer PEG-PASP with PZACH_2OH was based on that of



Scheme 1. Synthesis of the polymeric prodrug.

Yokoyama *et al.* [5, 10, 11], in which an ester bond is formed between the OH on the ligand and the free carboxyl group on aspartyl residues, in the presence of the condensing agent EDC (Scheme 2). In order to ensure that the end product PEG-PASP-PZA was indeed formed by a covalent bond between the carrier and the drug, some tests were adopted, involving dialysis. When the product was dialyzed in a low exclusion limit (1 kDa) membrane, any unbound drug would be expected to be washed out. If the drug were still associated with the polymer, it might possibly be trapped physically in the pores of the polymer matrix; to test this, the carrier copolymer was mixed with the drug and the mixed solution dialyzed under the same conditions as the end product. The dialysis water was changed every 30 min during the four-hour experiment and all these extracts were lyophilized, as was the product that remained inside the membrane. Each residue was submitted to TLC, with the free drug and the carrier as standards. The chromatographic spots corresponding to the drug began to appear in the



Scheme 2. Condensation reaction of copolymer PEG-PASP with $PZACH_2OH$.

first extract of both the derivative and the mixture. After four extracts, no more drug was dialyzed from the derivative, whereas it was washed out of the mixture more quickly, practically disappearing after two extracts.

The ^{1}H- and ^{13}C-NMR spectra confirmed the end product. However, shifts that were not seen with the copolymer appeared with the product, characterizing it as PEG-PASP-PZA, since the main shifts observed, aside from those seen with the precursor, were those of the bound drug. A stronger indication that the drug was bonded to the copolymer came from the observation that the copolymer, in isolation or mixed with the drug, failed to produce the light-scattering seen with the micellar end product. The proportion of free carboxyl groups present in the PEG-PASP-PZA, determined by acid-base volumetric analysis, showed that the great majority of them (86%) had been esterified by the tuberculostatic substituent.

Micelles can be described in terms of several characteristics, such as: critical micelle concentration (CMC), hydrodynamic or micelle radius, aggregation number, degree of dissociation, among others. When the CMC is reached in a series of solutions of an amphiphilic micelle-forming molecule, some physicochemical properties of the solution undergo an abrupt change. Thus, to obtain the value of the CMC, properties such as the solubility of dyes, surface tension, and conductivity are observed over a range of concentrations. While it must be kept in mind when analyzing these data that the CMC is

not strictly a single point but a concentration band, in several cases it has been found to be a sufficiently narrow band to be treated as a point [17].

When the modified pyrazinamide was condensed with the poly(ethylene glycol)-poly(aspartic acid) copolymer, a micelle-forming derivative was obtained that was characterized in terms of its CMC and micelle diameter. The first of these values was obtained by the dye-solubility technique and the second by light-scattering. The CMC was found by observing the solubility of Sudan III in PEG-PASP-PZA solutions of various concentrations, chosen by trial and error to cover the range 5.0×10^{-7} to 5.0×10^{-3} mg/L. The absorbance due to the dye was practically constant at concentrations of the polymer derivative up to a critical value, while above this point the absorbance-concentration curve showed a sharp rise in slope, indicating that micelles were formed and that Sudan III, insoluble in water, was taken up by these micelles and, hence, colored the solution. The sharp bend in the curve, at approximately 5.0×10^{-3} mg/L, was therefore the CMC for PEG-PASP-PZA (Fig. 1). The mean diameter of PEG-PASP-PZA micelles, obtained by analyzing the dynamic light-scattering (DLS) data, was 78.2 nm. Many of the preparations of micelle-forming derivatives, however, exhibited mono-, bi-, or trimodal size distribution profiles according to the DLS data, with mean diameters that were much larger than would be desirable in the prodrug. This was observed despite the careful control of temperature and stirring during the synthesis of PEG-PBLA. It was assumed that if the degree of polymerization of NCA-BLA in PEG-PBLA was very high, this would damage the formation of small micelles for drug delivery, as planned. In order to overcome this problem, in addition to the strict control of reaction conditions, post-synthetic procedures were implemented: sonication of the polymeric product, elution of the mixed polymer through Sephadex® columns, with distilled water as the mobile phase, and ultrafiltration through a YM3 43 mm 10 PK membrane. In this way, smaller micelles were produced.

Two hypotheses were put forward to explain the multimodal distribution of micelle sizes: (1) very large molecules of PEG-PASP-PZA were mixed with the much smaller ones that led to useful micelles, so that the micelles were very big on average; (2) the apparent size distribution represented micelle aggregates and not single micelles. With the aim of separating lower molecular weight components, the micelle-forming derivative was dialyzed against distilled water, in a membrane of exclusion cut off 12–14 kDa. End product molecules in the dialyzate would possibly form smaller micelles and, even if they aggregated, the aggregates would be smaller.

The fact that the molecular mass of the polymer was unknown made it hard to choose the best dialysis membrane to separate the micelles. The actual choice took account of the fact that the copolymerization started with a high molecular weight (5 kDa) polymer, CH_3-PEG-NH_2. Nevertheless, some positive results were achieved with the dialyzed fractions.

Considering the mass of pyrazinamide incorporated on 86% of the carboxyl groups on carrier, at the respective concentration that PEG-PASP-PZA showed activity against Ra and Rv of *M. tuberculosis*, corresponding to a 0.475 and 0.950 μg/mL of pyrazinamide, it shows a superior activity when comparing to PZA (6.25 and 12.5 μg/mL) for the same strains.

Table 1 shows the minimum inhibitory concentrations (MIC) of compounds and the amount of pyrazinamide incorporated on polymer according to degree of substitution, which is determined by the ratio between free and substituted carboxyl groups on polymer used for the prodrug synthesis.

Even though we still not realize the assays that show how the pyrazinamide derivate acts, Yokoyama *et al.* introduce three hypothesis about micelle-forming polymeric drugs and its mechanism of action, which are: the drug is released to interact with the target, without any micelle participation, and in this specific case, that is a prodrug; or the micelle interacts directly with the target; or from a controlled equilibrium, the micelle form remains in a balance with a single chain from the polymeric derivate, which would be responsible for the activity.

Due to the reduced diameter and the hydrophilic surface constituted by low density PEG derivate, the PZA derivate can present reduced renal filtration and capture by reticule endothelial system. Besides that, it can prolong duration time on blood circulation because of its structure that connects a hydrophobic chain and another hydrophilic, which award to the copolymeric block a higher thermodynamic stability, and consequently, lower critical micelle concentration, allowing its use for long periods, in very dilute conditions, as in blood fluid, making this a promising carrier for pyrazinamide, capable to form micelles.

Experimental

Chemistry

Pyrazinamide was obtained from FURP (Fundação para o Remédio Popular), α-metil-ω-amineoxiethylene (MW 5,000) was purchased from Shearwater, Inc., USA, and EDC (1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride) from

Aldrich. Solvents and others reagents were obtained from different commercial sources.

Analytical methods

IR absorption spectra in the range of 4000 to 400 cm^{-1} were obtained from samples in KBr pellets with a Shimadzu spectrophotometer. ^{13}C- and ^{1}H-NMR spectra were collected in the Advance DPX spectrometer (Bruker) at 300 and 500 MHz, respectively, using 5 mm diameter resonance tubes, with D_2O, DMSO-d_6. Melting ranges of products were measured, without correction, in an Electrothermal melting-point apparatus. Percentages of C, H, and N in compounds were determined in the Elemental Analyser 24013 CHN (Perkin-Elmer). Analytical TLC was used to monitor the purification of polymeric derivatives and bound drug. Silica-gel coated 60 F_{254} aluminum plates were used, with $CHCl_3$: CH_3OH (1 : 1 v/v) as the eluent.

Determination of free carboxylic groups

The fraction of free –COOH groups in aqueous solutions of PEG-PASP copolymer and its derivative PEG-PASP-PZA was determined by titration against 0.01 M NaOH, with phenolphthalein as indicator.

Visible absorption spectrophotometry

The critical micelle concentration (CMC) was found by analyzing the apparent solubility of the dye Sudan III in aqueous solutions of the pyrazinamide-substituted block copolymer of poly(ethylene glycol)-poly(aspartic acid). Solutions of PEG-PASP-PZA were prepared at concentrations from 5.0×10^{-7} to 5.0×10^{-3} mg/L and 10 mg Sudan III was added to each solution. Solution absorbances at 519 nm were determined in a 1 cm optical cell in a Shimadzu UV1601PC spectrophotometer.

Dynamic light-scattering (DLS)

An aqueous solution of PEG-PASP-PZA (1% w/v) was submitted to ultrafiltration through the Amicon YM3 43 mm 10 PK membrane, and then the diameter of the micelle of PZA-polymer derivative was measured by DLS in a Malvern 4,700 MW system, equipped with a 60 mW He/Ne laser operating at 632.8 nm (Spectra-Physics 107) and a Brookhaven System thermostat-controlled bath.

Methods of purification

Lyophilization – Water was removed from samples in L4KR and MLW-LGAO5 Edwards lyophilizers.

Dialysis – Solutions were dialyzed against distilled water and/or acetate buffer, through a benzoylated dialysis membrane (exclusion limit 1.0 kDa), to remove the free tuberculostatic drug and any impurities of molecular weight lower than 1000. To separate the micelles of the pyrazinamide carrier PEG-PASP-PZA from larger polymer molecules or aggregates, dialysis against water was carried out with a membrane of an exclusion limit of 12 – 14 kDa.

The reactions involved in the synthesis of the polymeric prodrug derived from pyrazinamide are outlined in Scheme 1.

β-Benzyl-L-aspartate (BLA) [15]

Anhydrous ethyl ether (100 mL) was cooled in an ice bath and 10 mL of 95 – 98%(wt) sulfuric acid was added, followed by 100 mL (0.966 mol) benzyl alcohol (**2**). When the ether had been removed by evaporation at reduced pressure, 14.3 g (0.108 mol) aspartic acid (**1**) was added in small portions, with magnetic stirring. After 24 h of reaction, the mixture was maintained at room temperature while 200 mL ethanol were added, followed by 50 mL pyridine, the latter being added dropwise and the solution well shaken between each drop, until it turned opalescent. The mixture was kept at –30°C for 12 h and the precipitate formed was filtered and retained. It was recrystallized from water by adding drops of pyridine and then lyophilized, giving a white, odorless powder.

Yield: 43%; melting range: 218 – 220°C; elemental analysis (%): C 59.27, H 5.67, N 6.31 (cf theoretical (%): C 59.20, H 5.83, N 6.28). IR (KBr, cm^{-1}): 3050 (ν OH), 2750 (ν CH), 1735 (ν CO), 1691 ν CO), 1653 – 1514 (ν CH=$CH_{Ar.}$), 736 – 696 (δ CH_{Ar}). ^{1}H-NMR (DMSO-d_6) δ: 7,36 (m, H-7-11, 5H), 5.17-5.08 (l, H-5, 2H), 4.20-4.16 (t, H-2, 1H), 3.09-3.05 (d, H-3, 2H). ^{13}C-NMR (DMSO-d_6) δ: 169.7 (C_1), 169.4 (C_4), 135.7 (C_6), 128.5 (C_9 & C_{10}), 128.2 (C_7 & C_8), 128.1 (C_{11}), 66.3 (C_5), 48.6 (C_2), 34.3 (C_3).

β-Benzyl-L-aspartate-*N*-carboxyanhydride (NCA-BLA) [14]

Phosgene (**4**) was bubbled through a magnet-stirred suspension of 30 g (0.134 mol) of BLA in 300 mL anhydrous dioxane at 60°C, until the solid was dissolved. Excess phosgene and the dioxane were removed by a flow of N_2 at 40°C. A mixture of ethyl acetate and petroleum ether (1 : 1 v/v) was added to the remaining product, which was collected on a filter. The product was taken up in chloroform and 2-propanol (1 : 1 v/v) heated to 40°C and the mixture chilled at –30°C for 12 h, after which the solid was filtered and dried under reduced pressure, giving a white to brownish-yellow, odorless powder (**5**).Yield: 52%; melting range: 127 – 128°C; elemental analysis (%): C 57.77, H 4.67, N 5.31 (cf theoretical (%): C 57.83, H 4.42, N 5.62). IR (KBr, cm^{-1}): 3309 (δ NH), 2854 (ν CH), 1863 (ν CO), 1787 (ν CO), 1728 (ν CO), 1604 – 1456 (ν CH=CH_{Ar}), 758 (δ CH_{Ar}). ^{1}H-NMR (DMSO-d_6) δ: 7.36 – 7.30 (m, H-4-8, 5H), 5.12 (l, H-10, 1H), 4.18 (brs, H-2, 2H), 3.05 (brs, H-9, 2H). ^{13}C-NMR (DMSO-d_6) δ: 170.7 (C_1), 168.8 (C_{11}), 152.8 (C_{12}), 136.1 (C_3), 128.5 (C_8), 128.1 (C_6 & C_7), 128.0 (C_5 & C_4), 65.9 (C_2), 48,7 (C_{10}), 37.1 (C_9).

Poly(ethylene glycol)-poly(β-benzyl-L-aspartate) (PEG-PBLA) [7, 11, 12, 14]

NCA-BLA (**5**) 8.0 g (0.0321 mol) was dissolved in 12 mL *N*,*N*-dimethylformamide (DMF). To this solution, 106 mL double distilled chloroform was added, followed by 3.3 g (0.00066 mol) of α-methyl-ω-amino poly(oxyethylene) (CH_3-PEG-NH_2) (**6**) in 3.9 mL of similar chloroform. The reaction mixture was stirred for 24 h at 35°C, under a flow of N_2 or argon, finally sonicated for 30 min, and then poured into ether (alternatively, ethanol-water, 1 : 1 v/v) until the polymer precipitated. This was filtered, dried, redissolved in chloroform, and precipitated again with 2-propanol at 40°C. The final product was centrifuged at 3000 rpm for 5 min and the pellet retrieved in chloroform, filtered, and lyophilized to give a white to brownish-yellow odorless semi-solid (**7**). Yield: 6.54 g. ^{1}H-NMR (DMSO-d_6) δ: 8.26 (s, CON*H*) 7.32 (l, H-10-14), 5.08 (brs, H-8), 4.62 (brs, C$H_{\alpha\text{-amide}}$, NH_2), 3.44 (brs, H-2,3), 2.87 (s, H-1), 2.71 (s, C$H_{\alpha\text{-amide}}$). ^{13}C-NMR (DMSO-d_6) δ: 170.9 (C_7), 163.1 (C_4), 136.4 (C_9), 129.2 (C_{12}), 128.9 (C_{11} & C_{14}), 128.7 (C_{10} & C_{13}), 70.6 (C_2), 66.8 (C_8), 50.6 (C_1), 36.6 (C_6), 34.7 (C_3).

Poly(ethylene glycol)-poly(aspartic acid) block copolymer (PEG-PASP) [7, 11, 12, 14]

PEG-PBLA (**7**) (4.94 g) was dissolved in 49 mL stirred chloroform at 0°C. Next, 63 mL of 0.43 mol NaOH, dissolved in a mixture of water, 2-propanol, and methanol (1 : 2 : 2 v/v/v), was added. After 10 min reaction, the mixture was neutralized with acetic acid and the whole poured into 65 mL of ether. The resulting precipitate was filtered, redissolved in water and dialyzed against distilled water, firstly for 4 h in a dialysis membrane with exclusion limit 1.0 kDa and then in a second, with exclusion limit 12–14 kDa, for an equal time. The product was then lyophilized to a white to brownish-yellow odorless semi-solid (8). Yield: 3.01 g. Number of free carboxylic groups on the copolymer, available for reaction with $PZACH_2OH$, was determined as 5.8×10^{-4} mol COOH per g PEG-PASP. ^{1}H-NMR (D_2O) δ: 4.35 (s, $CH_{\alpha\text{- \& }\beta\text{-amide}}$), 3.59 (m, H-2,3), 3.27 (s, H-1), 2.64 (brs, $CH_{2\ \alpha\text{-amide}}$). ^{13}C-NMR ($D_2O$) δ: 177.8 ($C_{11}$), 175.6 ($C_8$), 168.8 ($C_7$), 70.1 ($C_2$), 63.0 ($C_5$), 62.0 ($C_1$), 32.8 ($C_3$, C_6, C_9), 55.2 (C_5).

Hydroxymethylpyrazinamide ($PZACH_2OH$) [15]

Pyrazinamide (**9**) (6.0 g, 0.0487 mol) was suspended in 30 mL water and 20 mL of 4 wt% aqueous K_2CO_3 solution added, followed by 20 mL of 38 wt% formaldehyde (**10**). The mixture was stirred for 20 h at room temperature, a further 50% of the formaldehyde was added and the mixture stirred for 4 h, at room temperature and filtered. The filtrate was evaporated to dryness at reduced pressure. The yellow solid residue was dissolved in water-acetone (10 : 90 v/v), recrystallized and dried to give a white to brownish-yellow odorless powder (11). Yield: 1.65 g (80%); melting range 126–128°C. ^{1}H-NMR (DMSO-d_6) δ: 8.94 (s, H-2, 1H), 8.67–8.63 (d, H-4, 1H), 8.20-8.30 (d, H-3, 1H), 4.96 (s, H-6, 2H). ^{13}C-NMR (DMSO-d_6) δ: 163.9 (C_5), 145.6 (C_4), 144.5 (C_3), 144.4 (C_2), 128.2 (C_1), 63.4 (C_6).

Poly(ethylene glycol)-poly(pyrazinamidomethyl aspartate) copolymer (PEG-PASP-PZA) [7, 11, 12, 14]

Hydroxymethylpyrazinamide (0.197 g, 0.0487 mol) was dissolved in 1.20 mL DMF. The condensing agent 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride EDC (0.82 g, **12**) was added to a solution of 2.30 g PEG-PASP in 20 mL water. The second solution was poured into the first and the ensuing reaction was carried out at 0°C for 4 h, with magnetic stirring. Another aliquot of EDC was added and the solution stirred for 24 h, at room temperature. The solution produced was dialyzed against acetate buffer (0.1 M, pH 4.5) for 4 h, in a membrane of exclusion limit 1.0 kDa, and then against distilled water, for an equal period. Finally, it was lyophilized, leaving a brownish-yellow odorless semi-solid (**13**). Yield: 1.65 g. Taking the estimated number of free carboxylic groups in PEG-PASP to be the true total, the percent substitution of these groups by $PZACH_2OH$ was 85.7% (4.9706×10^{-4} mol). ^{1}H-NMR (D_2O) δ: 8.63–8.57 (l, H-14, H-16, H-17), 5.65 (brs, H-12), 4,87 (s, $CH_{\alpha\text{- \& }\beta\text{-amide}}$), 3.50 (m, H-2, H-3), 3.01 (s, H-1), 2.87 (brs, H-9), 2.72 (brs, H-6). ^{13}C-NMR (D_2O) δ: 172.3 (C_7), 170.9 (C_{11}), 168.6 (C_8), 162.7 (C_4, C_{13}), 147.6 (C_{17}, C_{16}), 144.8 (C_{15}), 143.5 (C_{14}), 69.8 (C_2), 62.7 (C_{10}), 52.2 (C_1), 39.9 (C_3).

Biological method

Microbiological *in vitro* assay – This assay was performed with the free drug, the synthesized micellar prodrug derived from it (PEG-PASP-PZA) and the carrier (PEG-PASP). The MICs of each substance for *M. tuberculosis* standard strains H_{37}Ra – 25177ATCC and H_{37}Rv – 27294ATCC (denoted Ra, Rv) were estimated by the microplate Alamar Blue assay, proposed by Franzblau *et al.* [16]: the strains were cultured in broth containing serial dilutions of test compounds in sealed wells in a 96-well microplate. After 5 days at 37°C, a solution of Alamar Blue, a redox dye that is reduced from blue to pink by growing *M. tuberculosis* cells, was added to the wells, which were resealed and incubated for a further 24 h. The MIC was the lowest concentration of a substance that prevented the well changing from blue to pink.

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