Journal of Controlled Release 177 (2014) 84–95

Contents lists available at ScienceDirect

Journal of Controlled Release

journal homepage: www.elsevier.com/locate/jconrel

Use of poly(amidoamine) drug conjugates for the delivery of antimalarials to *Plasmodium*

Patricia Urbán [a,b,c], Juan José Valle-Delgado [a,b,c], Nicolò Mauro [d], Joana Marques [a,b,c], Amedea Manfredi [d], Matthias Rottmann [e], Elisabetta Ranucci [d], Paolo Ferruti [d], Xavier Fernàndez-Busquets [a,b,c,*]

[a] Nanomalaria Group, Institute for Bioengineering of Catalonia (IBEC), Baldiri Reixac 10-12, ES-08028 Barcelona, Spain
[b] Barcelona Centre for International Health Research (CRESIB, Hospital Clínic-Universitat de Barcelona), Rosselló 149-153, ES-08036 Barcelona, Spain
[c] Biomolecular Interactions Team, Nanoscience and Nanotechnology Institute (IN2UB), University of Barcelona, Martí i Franquès 1, ES-08028 Barcelona, Spain
[d] Department of Chemistry, Università degli Studi di Milano, Via Golgi 19, IT-20133 Milano, Italy
[e] Swiss Tropical and Public Health Institute, Socinstrasse 57, PO Box, CH-4002 Basel, Switzerland

ARTICLE INFO

Article history:
Received 6 November 2013
Accepted 21 December 2013
Available online 7 January 2014

Keywords:
malaria
nanomedicine
Plasmodium
polyamidoamines
polymer–drug carriers
targeted drug delivery

ABSTRACT

Current malaria therapeutics demands strategies able to selectively deliver drugs to *Plasmodium*-infected red blood cells (pRBCs) in order to limit the appearance of parasite resistance. Here, the poly(amidoamines) AGMA1 and ISA23 have been explored for the delivery of antimalarial drugs to pRBCs. AGMA1 has antimalarial activity per se as shown by its inhibition of the *in vitro* growth of *Plasmodium falciparum*, with an IC_{50} of 13.7 μM. Fluorescence-assisted cell sorting data and confocal fluorescence microscopy and transmission electron microscopy images indicate that both polymers exhibit preferential binding to and internalization into pRBCs versus RBCs, and subcellular targeting to the parasite itself in widely diverging species such as *P. falciparum* and *Plasmodium yoelii*, infecting humans and mice, respectively. AGMA1 and ISA23 polymers with hydrodynamic radii around 7 nm show a high loading capacity for the antimalarial drugs primaquine and chloroquine, with the final conjugate containing from 14.2% to 32.9% (w/w) active principle. Intraperitoneal administration of 0.8 mg/kg chloroquine as either AGMA1 or ISA23 salts cured *P. yoelii*–infected mice, whereas control animals treated with twice as much free drug did not survive. These polymers combining into a single chemical structure drug carrying capacity, low unspecific toxicity, high biodegradability and selective internalization into pRBCs, but not in healthy erythrocytes for human and rodent malarias, may be regarded as promising candidates deserving to enter the antimalarial therapeutic arena.

1. Introduction

More than 40% of the world's population lives at risk of contracting malaria. The most recent estimates indicate several hundred million clinical cases and 660,000 deaths in 2010 [1,2], of which the large majority are children below 5 years old [3,4]. The recent call for elimination and eradication of the disease requires research from multiple fronts, including developing strategies for the efficient delivery of new medicines [5]. Five *Plasmodium* species cause disease in humans, namely, *P. vivax*, *P. ovale*, *P. malariae*, *P. knowlesi* [6] and *P. falciparum*, with the latter being responsible for the most deadly and severe cases. When taking a blood meal, the female *Anopheles* mosquito inoculates *Plasmodium* sporozoites that in the liver infect hepatocytes and proliferate into thousands of merozoites [7]. Merozoites invade red blood cells (RBCs), where they build a parasitophorous vacuole inside which the parasite develops first into rings, and then into the late forms trophozoites and schizonts. Schizont-infected RBCs burst and release more merozoites, which start the blood cycle again. Because the blood-stage infection is responsible for all symptoms and pathologies of malaria, *Plasmodium*-infected RBCs (pRBCs) are a main chemotherapeutic target [8].

Since antimalarial drug delivery currently relies on compounds with little or no specificity for pRBCs, the administration of most drugs requires high doses. However, the unspecificity of toxic drugs demands low concentrations to minimize undesirable side effects, thus incurring the risk of sublethal doses favoring the appearance of resistant pathogen strains [9]. Nanomedicine, which uses nanosized tools for the treatment of disease [10], can fulfill the objective of achieving the intake of total amounts sufficiently low to be innocuous for the patient, but locally still lethal for the parasite. The development of novel delivery approaches is less expensive than finding new antimalarial drugs and may optimize their rate of release [11]. Current immunoliposomal prototypes engineered for the delivery of antimalarial drugs specifically to pRBCs [12,13] rely on antibody targeting and contain special lipids, making their synthesis too expensive for their practical widespread use in the routine treatment of most malaria cases, which are in developing areas with low per capita incomes. An essential aspect for the successful development of antimalarial nanomedicines resides on the choice of targeting elements, of which it has to be considered their

* Corresponding author at: Nanomalaria Unit, Centre Esther Koplowitz, 1st floor, CRESIB, Rosselló 149-153, ES-08036 Barcelona, Spain.
E-mail address: xfernandez_busquets@ub.edu (X. Fernàndez-Busquets).

0168-3659/$ – see front matter © 2014 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jconrel.2013.12.032

biocompatibility, cell specificity, binding affinity, ease of modification and conjugation to the drugs, production cost, scalability and stability in mass production. Polymers offer virtually unlimited diversity in chemistry, dimensions and topology, rendering them a class of materials that is particularly suitable for applications in nanoscale drug delivery strategies [14].

Poly(amidoamine)s [15,16] (PAAs) are a family of synthetic polymers exhibiting a combination of properties imparting them a potential in the biomedical field. They can be designed to be biocompatible and bioeliminable, and degrade to oligomeric products in aqueous media within days or weeks, depending on their structures [17–19]. Three PAAs were selected for the present study, namely, AGMA1, ISA23 and ISA1 (Fig. 1 and Supplementary data, Table S1). AGMA1 is obtained by polyaddition of 4-aminobutylguanidine (agmatine) with 2,2-bis(acrylamido)acetic acid and contains *tert*-amine, carboxyl and guanidine groups. It is amphoteric with isoelectric point of 10.0 and therefore at pH 7.4 is prevailingly cationic with, on average, 0.55 excess positive charges per unit. ISA23 is obtained by polyaddition of 2-methylpiperazine with 2,2-bis(acrylamido)acetic acid. Notwithstanding carrying two *tert*-amine groups and one carboxyl group per unit, it has isoelectric point of 5.5, being prevailingly anionic at pH 7.4 with, on average, 0.38 excess negative charges per unit. ISA1 is obtained by polyaddition of bis(acryloyl)piperazine with 2-methylpiperazine and bis(hydroxyethylethylenediamine). It is a rather weak polymeric base with, on average, 0.55 positive charges per unit at pH 7.4. All these polymers have been reported as vectors for the intracellular delivery of nucleic acids [20–22]. ISA1 and ISA23 have been also studied for protein delivery [22–24] and as anticancer drug carriers [25,26]. ISA23 had proven endowed with stealth-like properties and did not selectively concentrate in the liver [27], whereas a significant portion of AGMA1 showed hepatic localization after intravenous injection in mice [28]. pRBCs are known to be permeable to high molecular mass solutes [29] including peptides and proteins, with which PAAs share some features, such as the polyelectrolyte behavior and the presence of amide groups in the main chain. This led us to explore the potential of ISA1, ISA23 and AGMA1 as antimalarial drug carriers.

2. Materials and methods

Unless otherwise indicated, all reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.1. Synthesis of ISA1, ISA23 and AGMA1 with different molecular weight

Linear ISA23, [26] ISA1 [26] and AGMA1 [29] were synthesized as previously described, but prolonging the reaction time to 7 days. They were subsequently fractionated by sequential ultrafiltration through membranes (Amicon, Millipore) with decreasing nominal molecular weight cutoffs, namely, 100,000, 30,000, 10,000, 5,000 and 1,000 Da, in order to obtain for each polymer four fractions identified with a letter in alphabetical order for increasing molecular weight (e.g., for the AGMA1 series, the molecular weight follows the trend AGMA1-a < AGMA1-b < AGMA1-c < AGMA1-d).

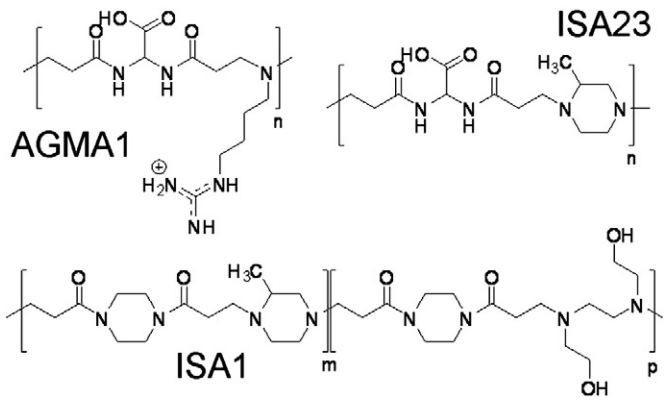

Fig. 1. Chemical structures of AGMA1, ISA1 and ISA23.

2.2. Synthesis of FITC-labeled AGMA1 and ISA23

FITC-labeled AGMA1 ($\overline{M}_n$ = 15,200, $\overline{M}_w/\overline{M}_n$ = 1.05) was prepared as previously reported [28]. Briefly, an AGMA1 sample carrying amine groups (AGMA1-NH_2) was prepared by substituting 7% on a molar basis of mono(*tert*-butoxycarbonyl)ethylenediamine for agmatine and subsequently deprotecting with 5% hydrochloric acid. A pH 7.4 AGMA1-NH_2 solution (10 mg/ml) was then treated with excess of an FITC solution in methanol (0.2 mg/ml). The mixture was stirred overnight at room temperature and centrifuged to eliminate insoluble impurities. The resultant clear solution was then dialyzed against water (membrane with 2,000 Da nominal molecular weight cutoff) and the fluorescein-labeled polymer isolated by freeze-drying the retained portion, with a quantitative recovery. The conjugation of AGMA1-NH_2 with FITC was confirmed by NMR and fluorescence microscopy, and the efficiency of the labelling procedure determined by measuring the fluorescence intensity at λ_{ex} = 480 nm and λ_{em} = 520 nm of a solution of AGMA1-FITC versus a standard FITC solution of known concentration. FITC-labeled ISA23 ($\overline{M}_n$ = 10,900, $\overline{M}_w/\overline{M}_n$ = 1.17) was prepared by substituting 7% on a molar basis of N-Boc ethylenediamine for 2-methylpiperazine in the preparation recipe and then deblocking and treating the resultant product as reported above for AGMA1-FITC. On a molar basis relative to the polymer repeating units, FITC content was 7.2% and 6.5% for AGMA1-FITC and ISA23-FITC, respectively.

2.3. Preparation of ISA23- and AGMA1-drug salts

Primaquine (PQ) free base was prepared as reported elsewhere [30]; briefly, a 10-mg/ml primaquine diphosphate aqueous solution was brought to pH 12 by the addition of 0.1 M NaOH, whereby PQ free base separated as an oil that was extracted (4 × 50 ml) with ether. The combined ether extracts were dried over Na_2SO_4 and evaporated in vacuo, leaving PQ as a dark brown oil with a yield of 50.9% w/w. Chloroquine (CQ) free base was prepared following the same procedure described for PQ, except for the initial dissolution of CQ diphosphate in an emulsion of 1:1 H_2O/ether. Then, CQ free base was recovered, after separation and evaporation of ethereal phase, as a white solid with a yield of 59.1% w/w. An ISA23 sample with $\overline{M}_n$ = 29,800 and $\overline{M}_w/\overline{M}_n$ = 1.28 was prepared as previously reported [27], but with a reaction time of 10 days, and brought to its isoelectric point (pH 5.4). A methanol solution of PQ or CQ free base (0.1 mg/ml) was added drop-wise to a solution of the polymer (10 mg/ml) until pH 7.4 was reached. The resultant clear solution was freeze-dried and the polymer salt retrieved as a white powder. The lyophilized product was perfectly soluble in water whereas the drug, as free-base, was not. The drug payload, calculated from the amount of drug needed to reach pH 7.4, corresponded to 15.1% and 32.9% (w/w) of the product for PQ and CQ, respectively. An AGMA1 sample with $\overline{M}_n$ = 20,800 and $\overline{M}_w/\overline{M}_n$ = 1.38, prepared as reported above [31], was treated using the same procedure followed for the ISA23–drug conjugate, apart from the fact that the drug addition was performed until pH 7.4 was reached, even though the isoelectric point of AGMA1 was 10.4. The drug payload (w/w), calculated as mentioned above in the case of ISA23 salts, was 29.4% for PQ and 14.2% for CQ.

2.4. Size exclusion chromatography (SEC) and dynamic light scattering (DLS) analysis

SEC analysis was performed using a Knauer Pump 1000 equipped with a Knauer Autosampler 3800, TKSgel G4000 PX and G3000 PX TosoHaas columns connected in series, light scattering/viscometer Viscotek 270 Dual Detector and a refractive index detector Waters