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Original article

PEG–Ara-C conjugates for controlled release

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Abstract

The antitumour agent 1-β-D arabinofuranosilcytosyne (Ara-C) was covalently linked to poly(ethylene glycol) (PEG) in order to improve the in vivo stability and blood residence time. Eight PEG conjugates were synthesised, with linear or branched PEG of 5000, 10000 and 20000 Da molecular weight through an amino acid spacer. Starting from mPEG-OH or HO-PEG-OH, conjugation was carried out to the one or two available hydroxyl groups at the polymer's extreme. Furthermore, to increase the drug loading of the polymer, the hydroxyl functions of PEG were functionalised with a bicarboxylic amino acid yielding a tetrafunctional derivative and, by recursive conjugation with the same bicarboxylic amino acid, products with four or eight Ara-C molecules for each PEG chain were prepared. A computer graphic investigation demonstrated that aminoadipic acid was a suitable bicarboxylic amino acid to overcome the steric hindrance between the vicinal Ara-C molecules in the dendrimeric structure. In this paper we report the optimised conditions for synthesis and purification of PEG–Ara-C products with a low amount of remaining free drug, studies toward the hydrolysis of PEG–Ara-C and the Ara-C deamination by cytidine deaminase, pharmacokinetics in mice and cytotoxicity towards HeLa human cells were also investigated. Increased stability towards degradation of the conjugated Ara-C products, in particular for the highly loaded ones, improved blood residence time in mice and a reduced cytotoxicity with respect to the free Ara-C form was demonstrated.

Keywords: PEGylation; EPR effect; Ara-C; 1-β-D-Arabinofuranosylcytosine; Dendrimer; Antitumour drugs

1. Introduction

1-β-D Arabinofuranosylcytosine (Ara-C), used alone or in combination with other compounds, is one of the most effective antitumour agents in the treatment of various types of human tumours such as acute myelogenous leukaemia, colon, breast and ovary carcinoma [1]. Its rapid clearance is due to the enzymatic conversion to the inactive and more soluble Ara-U. Such conversion is operated by cytidine deaminase, mainly in liver and kidney; for this reason Ara-C is administered by continuous i.v. infusion or as frequent, high-dose, schedules [2].

So far many different approaches have been attempted to improve its stability in vivo such as combination therapy with cytidine deaminase inhibitors as tetrahydroirudine, which unfortunately did not significantly improve the cytotoxic efficacy [3]. Macromolecular derivatives and prodrugs, obtained by acylation of N^4 position of the nucleoside, have also been synthesised with the aim of increasing the biological activity through protection against deamination and alteration of pharmacokinetic properties [4–7]. In fact, compounds obtained by conjugation of antitumour drugs to high molecular weight polymers and polypeptides, are now representing a new and promising approach to chemotherapy. These conjugates may act as classical prodrugs while, taking advantage of the EPR effect, may better accumulate into tumour mass and cross the cell membranes by endocytosis to reach their intracellular targets [8–10].

In the present paper, we investigated the covalent conjugation of Ara-C to poly(ethylene glycol) (PEG), a water soluble and biocompatible polymer of low toxicity, having different composition and structure. Eight different products have been synthesised in which the drug was covalently bound to the polymer backbone through an amino acid

Abbreviations: AD, aminoadipic acid; Ara-C, 1-β-D arabinofuranosilcytosyne; tr-Ara-C, 5′-*O*-trityl 1-β-D arabinofuranosilcytosyne; AUC, area under the curve; Cl, clearance; Nle, nor-leucine; $t_{1/2}\alpha$, distribution half time; $t_{1/2}\beta$, elimination half time; V_d, distribution volume.

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spacer. The bond involves the N^4 amino group of Ara-C pyrimidine ring and the carboxylic group of an amino acid spacer.

Unfortunately PEG has a severe limitation in its poor loading capacity, having only two terminal functional groups at the end of polymer chain (or just one in the case of the most used monomethoxypoly(ethylene glycol) (mPEG-OH), which can be functionalised and conjugated to drug. In recent studies [11,12], such limitation was circumvented by coupling a bicarboxylic amino acid, aspartic acid, to the PEG. Such derivatization doubled the number of active groups of the original molecule of PEG and with recursive derivatization using the same method; it was possible to achieve a dendrimeric structure at each PEG's extremity. However, the authors encountered some problems in this study, namely the low reactivity of the bicarboxylic acids groups towards Ara-C binding [11,12]. This limit, attributed to steric hindrance occurring between to two molecules of Ara-C when they are conjugated to the neighbouring carboxylic moieties, was overcome by extending the dendrimer arms with an amino alcohol (H_2N–[CH_2–CH_2–O]$_2$–H). We approached the problem with a molecular modelling study to find out a more suitable bicarboxylic amino acid for the synthesis of the dendrimeric structure on which Ara-C might be linked to, with the least steric hindrance. Computer aided design suggested that aminoadipic acid could be the solution, because in this molecule the carboxylic groups are sufficiently separated to accommodate Ara-C without the need of spacer arms. The theoretical indications were confirmed by the experimental results, since an easy synthesis of the desired products could be achieved.

Many properties of PEG–Ara-C conjugates were also investigated and reported here, such as the hydrolytic stability in blood and in buffer solution at different pH values, the influence of the polymer carrier on degradation rate of Ara-C by cytidine deaminase, the in vitro inhibition of human cancer cell growth and the blood residence time. A partial report of these results were already reported in part at the 2000 Controlled Release Society 28th Annual Meeting [13] and at Fifth International Symposium on Polymer Therapeutics [14].

2. Chemistry

2.1. Synthesis of Ara-C conjugates

PEGs of different molecular weights (5, 10 and 20 kDa) and shapes (linear or branched) were used for the synthesis of macromolecular Ara-C prodrugs. The choice of PEG as carrier was justified by its well-known properties: high water solubility, biocompatibility, lack of toxicity, low immunogenicity and presence of one or two functionalisable hydroxyl groups.

In a first group of conjugates (**1–6**; Table 1), the syntheses were carried out by conjugation of PEG to Ara-C through an amino acid spacer (nor-leucine or lysine).

In the conjugates **4** and **5**, the amino acid lysine is a structural part of the branched PEG and the carboxylic group of lysine is used for conjugation. For monofunctional linear PEG–Ara-C (**1, 2, 3 6**), the amino acid nor-leucine (Nle) was used as spacer between polymer and drug, because we wanted comparable chemistry and structures (the presence of one amino acid as spacer arm) in both types of conjugates, the linear and the branched ones. Among the many possible monocarboxylic amino acids, Nle was chosen, since this was already proposed and used as spacer in PEG conjugation of peptides, proteins and non-peptide drugs for the special advantages presented by this amino acid in the analysis of conjugates [15–17]. The binding of PEG–amino acid carboxylic group to the primary aromatic amino group (N^4) of Ara-C would avoid the rapid in vivo enzymatic degradation of Ara-C to Ara-U.

The hydroxyl groups of PEG, after activation by *p*-nitrophenyl chloroformate [16], yielded a stable carbamate linkage between PEG and the amino acid. The degree of PEG hydroxyl group activation with *p*-nitrophenyl chloroformate, determined by UV analysis of the *p*-nitrophenol released from PEG–*p*-nitrophenyl carbonate after alkaline hydrolysis, was in the range of 92–96%. Activated PEG was coupled with the amino acid and the intermediate PEG-amino acid was conjugated to Ara-C by EDC/NHS activation [16] (see Fig. 1a,b).

Improved species of Ara-C conjugates (**7, 8**), with higher drug loading capacity, were finally obtained by conjugation

Table 1
Ara-C loading in the conjugates and percentage of unbound Ara-C present in the products

Conjugates	Total Ara-C [a] (wt/wt) (%)	Free Ara-C [b] (wt/wt) (%)
(**1**) $mPEG_{5000}$–Nle–Ara-C	4.12	0.73
(**2**) $mPEG_{10000}$–Nle–Ara-C	2.08	0.10
(**3**) $mPEG_{20000}$–Nle–Ara-C	1.05	0.20
(**4**) $mPEG2_{10000}$–Lys–Ara-C	2.15	0.12
(**5**) $mPEG2_{20000}$–Lys–Ara-C	1.02	0.19
(**6**) PEG_{10000}–$(Nle)_2$–$(Ara\text{-}C)_2$	3.91	0.36
(**7**) PEG_{10000}–$(AD)_2$–$(Ara\text{-}C)_4$	6.98	0.09
(**8**) PEG_{10000}–$(AD)_2$–$(AD)_4$–$(Ara\text{-}C)_8$	13.07	0.18

[a] Percentage referred to the weight of product.
[b] Percentage of unbound Ara-C as referred to the total Ara-C present in the product.

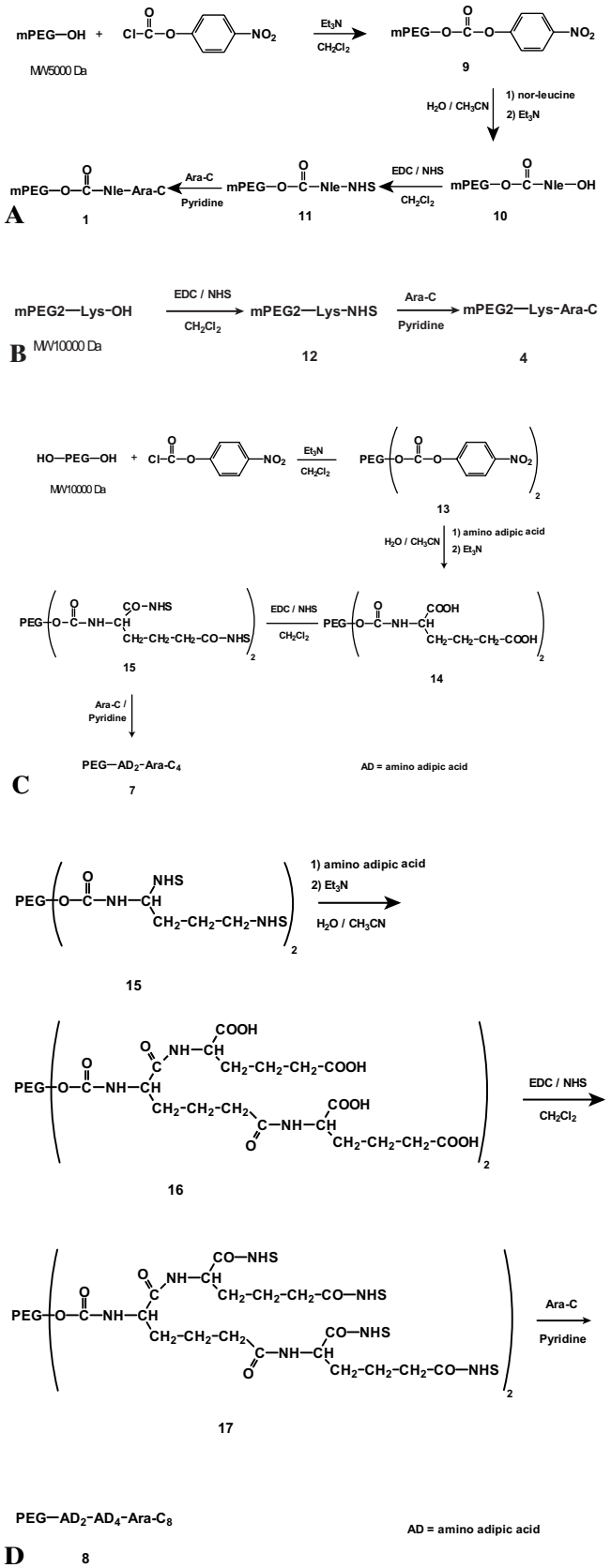

Fig. 1. (a) Chemical route of $mPEG_{5000}$–Nle–Ara-C (**1**). (b) Chemical route of $mPEG2_{10000}$–Lys–Ara-C (**4**). (c) Chemical route of PEG_{10000}–AD_2–Ara-C_4 (**7**). (d) Chemical route of PEG_{10000}–AD_2–AD_4–Ara-C_8 (**8**).

of tetrafunctional or octafunctional PEG to drug. Tetrafunctional PEG was synthesised through conjugation of a bicarboxylic amino acid (L-2-aminoadipic) to the two hydroxyl groups of PEG while, by a second conjugation step between L-2-aminoadipic and tetrafunctional PEG, an octafunctional PEG was obtained. These compounds were prepared using the same chemical route as above reported, while the L-2-aminoadipic was chosen as leading bicarboxylic acid after molecular modelling investigation (see Fig. 1c,d).

2.2. Computational studies

To investigate the capability of Ara-C to chemically react with PEG–bicarboxylic acid derivatives, a molecular modelling study was carried out on different simplified structures. Based on our hypothesis, the chemical reactivity of Ara-C depends upon its accessibility to the carboxylic acid functions present on the PEG–bicarboxylic acid derivatives. In the present study, we selected PEG-aspartic acid, the amino acid reported by other author [11,12] and PEG-aminoadipic acid as models of PEG–Ara-C conjugates with different dendrimeric organisation. We remind that in the case of aspartic acid a spacer between the carboxylic acid and Ara-C was needed to achieve a satisfactory degree of binding [11,12]. Moreover, to deeply analyse the steric requirements of Ara-C to react with both PEG–(aspartic acid) and PEG–(aminoadipic acid) conjugates, we have also modelled the functionalised derivatives PEG–(aspartic acid)–(aspartic acid)$_2$–(Ara-C)$_2$ and PEG–(aminoadipic acid)–(aminoadipic acid)$_2$–(Ara-C)$_2$ conjugates. An exhaustive conformational analysis, based on a "Stochastic Conformational Search Algorithm" was performed to sampling local minima of both the potential energy surfaces (see Section 5.2 for details). The most stable conformer for both PEG–(bicarboxylic acid)–(bicarboxylic acid)$_2$–(Ara-C)$_2$ conjugates is shown in Fig. 2. Analysing our theoretical models, it is clear that the dendrimeric organisation of PEG–aspartic acid conjugate presents high steric congestion and, consequently, this might be the reason of the shown low reactivity of the carboxylic acid functions. Indeed, the distance between the two carboxylic acid functions is around 6.3 Å. On the other hand, PEG–aminoadipic acid conjugate structure is characterised by a less steric congestion with the carboxylic acid functions far enough (ca. 9.4 Å) to easily accommodate the conjugation with Ara-C.

2.3. Characterisation of Ara-C conjugates

Several pieces of evidences indicated that acylation of the amino group took place with no involvement of the sugar OH group, namely:

(1) UV spectra of conjugates showed disappearance of the typical UV peak of free Ara-C (272 nm) and formation of the new peaks of the conjugated form of Ara-C (300, 247, 213 nm), which are due to acylation of Ara-C N^4 amino group (Fig. 3). The spectra of the

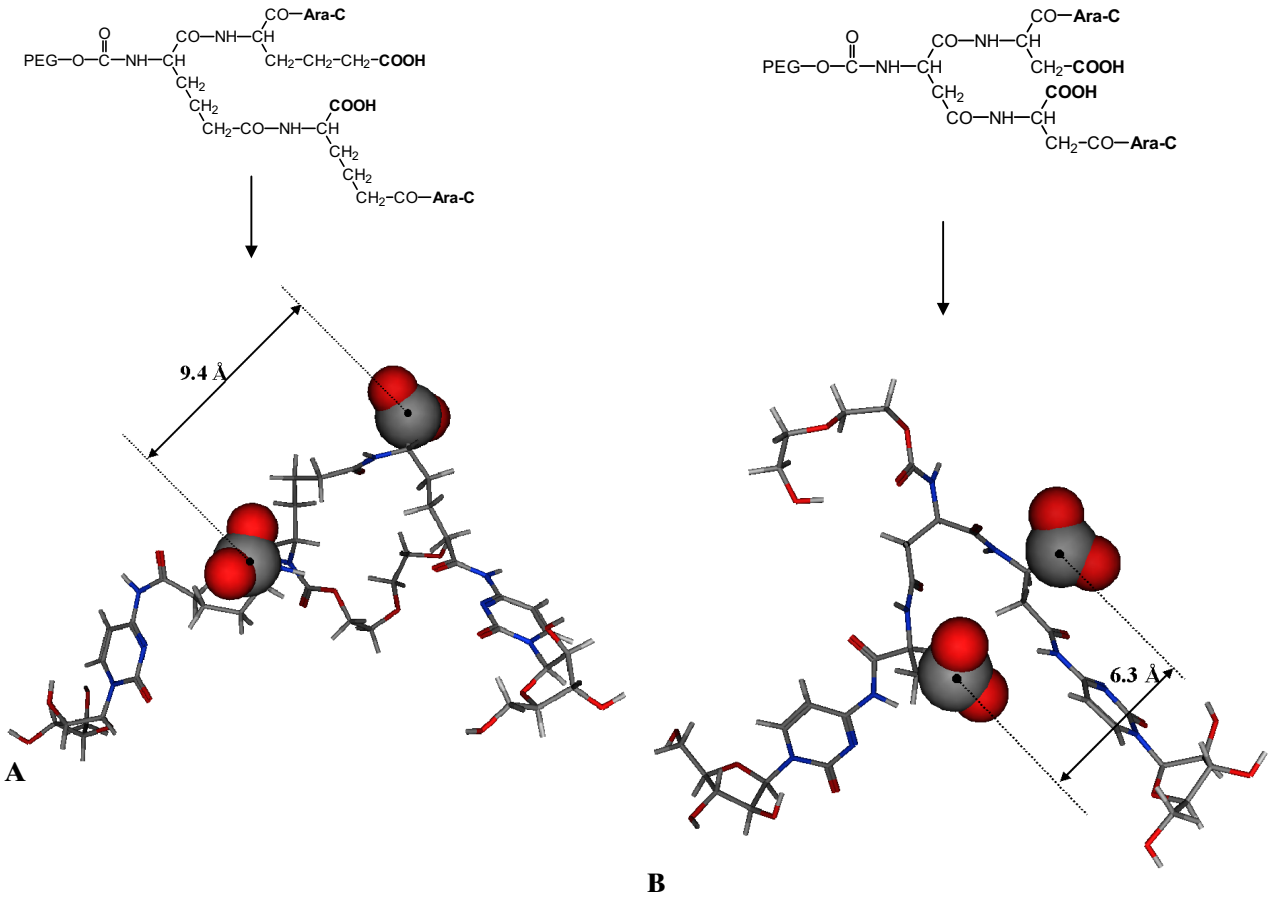

Fig. 2. (a) Molecular structure of the most stable conformation of PEG–(aminoadipic acid)–(Ara-C)$_2$ conjugate. The two carboxylic acid functions are emphasize by space filling representation, and the distance (Å) between them is reported. (b) Molecular structure of the most stable conformation of PEG–(aspartic acid)–(Ara-C)$_2$ conjugate. The two carboxylic acid functions are emphasise by space filling representation, and the distance (Å) between them is reported.

conjugates have shown the same peaks as those obtained using glutaric anhydride as acylating agent [4,7,19].

(2) Identical compounds, as verified by ^{1}H NMR and HPLC analysis, were obtained by direct PEGylation of Ara-C as well as by PEGylation of 5′-*O*-trityl–Ara-C (tr-Ara-C) followed by detritylation.

(3) The enzyme cytidine deaminase failed to convert the conjugated Ara-C in agreement with the *N*-acylation, as already demonstrated by Onishi et al. [4].

The ^{1}H NMR spectra for the PEG–Ara-C conjugates revealed the expected peaks of the –O–CH_2–CH_2– protons of PEG chain, of the amino acid moiety and of Ara-C.

The presence of free Ara-C in conjugates could be verified by RP-HPLC because products are eluted as single peak around t_R = 23 min whereas free Ara-C is eluted at t_R = 6.46 min (Fig. 4). On the other hand, the content of Ara-C in the conjugates was determined by RP-HPLC analysis of hydrolysed product samples. The hydrolysis was performed by incubation in NaOH 1 N [7], which released Ara-C from the conjugates. This evaluation method was necessary since bound Ara-C in conjugates has different UV extinction coefficient with respect to the free drug, which prevents a direct spectrofotometric evaluation.

Ara-C release from the conjugates was studied following incubation in aqueous solutions at various pHs at 37 °C. Fig. 5 reports the Ara-C release profile from monofunctional, linear (**1**) or branched (**5**), derivatives and from a tetrafunctional derivative one (**7**). Since, in general, macromolecules are transported into cell by endocytosis and accumulated into endosomes and lysosomes [9], the drug release at pH 6.0 can simulate the weakly acidic conditions of these compartments.

2.4. Biological studies of Ara-C conjugates

Three representatives PEG conjugates (**1, 5** and **7**) and unbound Ara-C were tested towards cytidine deaminase incubation (Fig. 6). This study allowed investigating the effective protection of conjugation against deamination at N^4 of Ara-C, because when this reaction takes place in vivo it leads to the completely inactive Ara-U.

To better verify the stability of conjugates as potential antitumour prodrugs they were investigated by incubation in plasma solution also (as models **1, 5** and **7** conjugates were studied).

Finally, in vitro cytotoxic activities of PEG–Ara-C derivatives were evaluated against HeLa human cells.

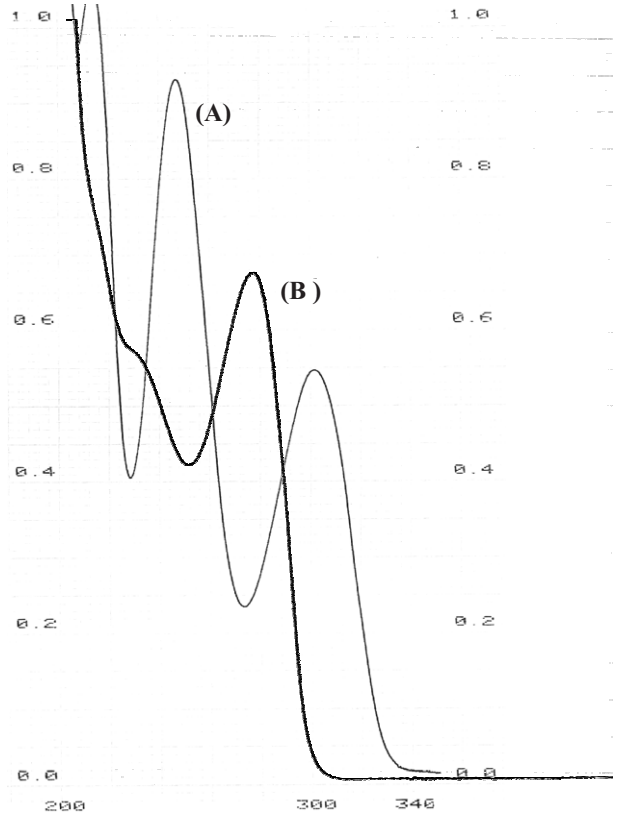

Fig. 3. UV spectres of $mPEG_{5000}$–Nle–Ara-C (**1**) (A) in comparison with that of Ara-C (B) in phosphate buffer 1/15 M pH 7.4.

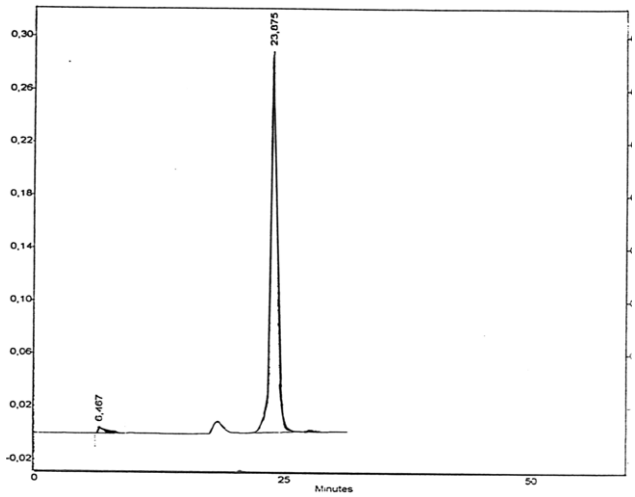

Fig. 4. Elution profile of $mPEG_{5000}$–Nle–Ara-C (**1**) (t_R = 23 min) obtained by C_{18} reverse phase in HPLC. Ara-C was present as impurity (0.73%) and eluted at t_R = 6.46 min.

2.5. Pharmacokinetics of Ara-C conjugates

To prove the improvement of PEG conjugation on Ara-C drug a pharmacokinetics study was performed in mice for the conjugates **1, 5, 7** and **8**. These compounds were chosen because, for their different polymer mass and shape, they can demonstrate the effect of these parameters in the in vivo behaviour.

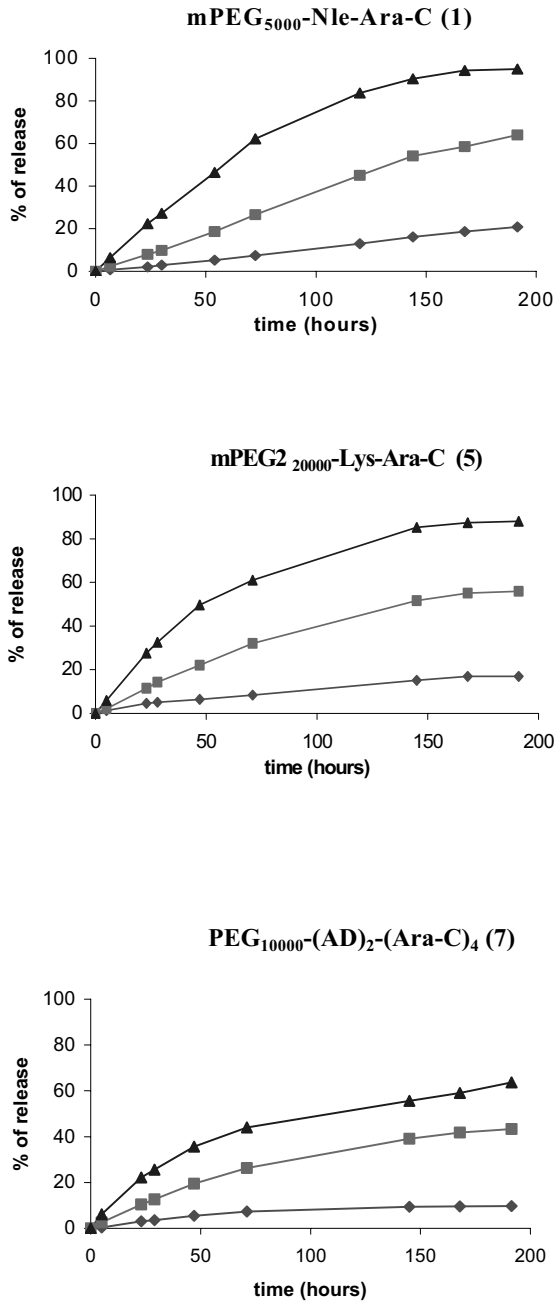

Fig. 5. Ara-C release profile from $mPEG_{5000}$–Nle–Ara-C (**1**), $mPEG2_{20000}$–Lys–Ara-C (**5**) and PEG_{10000}–$(AD)_2$–$(Ara\text{-}C)_4$ (**7**) at pH 8 (▲), pH 7.4 (■) and pH 6 (◆). The conjugates were incubated in 0.07 M phosphate buffer at 37 °C. The amount of Ara-C was monitored by reverse phase HPLC.

3. Results and discussion

The preparation of PEG–Ara-C conjugates was carried out through a chemistry that allows obtaining the wanted polymeric compounds with satisfactory yield and drug loading (Fig. 1a–d). After proper purification, the percentage of unbound Ara-C in the products was as low as 0.1–0.7% (Table 1). This amount of unbound drug is usually considered acceptable in polymeric prodrug preparation of antitumour agents. In addition to conjugates where the drug binding was

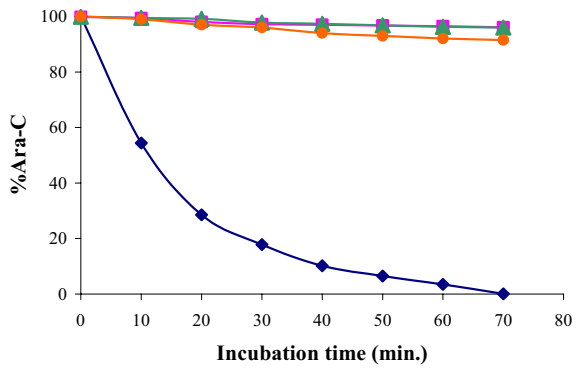

Fig. 6. Resistance of $mPEG_{5000}$–Nle–Ara-C (**1**) (■), $mPEG2_{20000}$–Lys–Ara-C (**5**) (▲), PEG_{10000}–$(AD)_2$–$(Ara\text{-}C)_4$ (**7**) (●) and Ara-C (◆) to cytidine deaminase. The disappearance of Ara-C was estimated by reverse phase HPLC.

limited to the lone reactive group of methoxy PEG (**1–5**) or to the two hydroxyl groups of PEG diol (**6**) a multi-functional compounds **7** and **8** were prepared. Such conjugates have the purpose of increasing the drug loading with respect to the polymer chain. We reached the goal by binding a bicarboxylic amino acid at the polymer's hydroxyl groups; such strategy allowed doubling of functionalisable groups at each conjugation step between polymer and bicarboxylic amino acid.

Analysing our theoretical models, it was clear that the dendrimeric organisation of PEG–aspartic acid conjugate (the branching amino acid used by others authors [11,12]) presents high steric congestion and, consequently, this might be the reason of the shown low reactivity of the carboxylic acid functions towards Ara-C [11,12]. On the other hand, PEG–(aminoadipic acid) structure, the one we exploited in the present study, is characterised by a less steric congestion with the carboxylic acid function, far enough to easily accommodate the conjugation with Ara-C (Fig. 2a,b).

PEG–Ara-C conjugates were investigated for their stability under different environmental conditions (Fig. 5). Incubation of conjugates in solution at different pH values demonstrated their stability at pH 6, since a release of 10–15% only after 8–10 days of incubation takes place. This result indicates that drug release in the acidic endosome and lysosome compartments cannot play a significant role in the activity of these prodrugs. On the other hand, the release of Ara-C was faster at 7.4 and pH 8.0 since it reached values of about 60% and 80%, respectively. It is noteworthy the influence of both polymer shape and drug loading in the rate of hydrolysis. For instance, at pH 7.4, the rate of release of Ara-C follows the compounds order: **1** =**5** >**7** indicating a role of hindrance in the hydrolytic stability. This reduction of hydrolysis may be due to the protecting effect of polymers, behaviour in agreement with other polymer-drug conjugates, such as dextran [18].

In general the conjugate was also found to protect toward the N^4 deamination of Ara-C in fact, when three representative PEG–Ara-C conjugates (**1, 5** and **7**) and unbound Ara-C were treated with cytidine deaminase (Fig. 6) for 70 h, only free Ara-C was completely deaminated to Ara-U. This result agrees with the study of Onishi et al. [4], who demonstrated that an acyl substitution on the N^4 of Ara-C might prevent enzymatic deamination.

The stability of the same three conjugates (**1, 5** and **7**) was also studied in plasma solution where degradation may occur by hydroxyl ions and enzymes. It was found that only 10% of conjugates were degraded giving rise to Ara-U after 8 h of incubation (data not shown). This percentage corresponds to the amount of conjugate hydrolysed in phosphate buffer at the same pH (see Fig. 5) indicating that Ara-C is first released from conjugates by a non-enzymatic hydrolysis, which in turn is converted to Ara-U by plasma enzymes.

In vitro cytotoxic activity of PEG–Ara-C derivatives was evaluated by HeLa human cells incubation. The results, referred to 24 h of incubation, demonstrated a much lower cytotoxicity of Ara-C conjugates than of free Ara-C. In fact it was shown that in the presence of free Ara-C at 100 μM concentration complete cell mortality was found whereas for the conjugates, at the same concentration the death value was ranging from 10% to 30% (data not shown). This lower cytotoxicity is probably due to the low amount of free Ara-C hydrolytically released from conjugates during the 24 h of incubation that cross the membrane by diffusion as free drug or, alternatively, to a low internalisation rate of the whole conjugates.

Pharmacokinetic studies, carried out in mice, demonstrated that conjugation of Ara-C with PEG significantly reduced the elimination rate of drug from blood. Table 2 reports relevant pharmacokinetic data for Ara-C and the four representative conjugates, **1, 5, 7** and **8**. These compounds were chosen in order to compare the effect of different polymer's mass and shape on drug release. It was demonstrated that compounds characterised by higher molecular weight, branched polymer chain (**5**) or branching at the level of the amino acid moiety (**7** and **8**) had a higher values of half life or AUC than the linear conjugate (**1**).

Table 2
Relevant pharmacokinetic values of Ara-C and of four PEG–Ara-C conjugates after i.v. administration to mouse

Conjugates	$t_{1/2}\alpha$ (min)	$t_{1/2}\beta$ (min)	AUC (μg min/ml)	Cl (ml/min)	V_d (ml)
Ara-C	1.2	14.4	2.4×10^2	1.02	21.20
$mPEG_{5000}$–Nle–Ara-C (**1**)	3.4	53.8	2×10^3	0.125	9.70
$mPEG2_{20000}$–Lys–Ara-C (**5**)	15.7	123.0	1.5×10^4	0.017	3.03
PEG_{10000}–$(AD)_2$–$(Ara\text{-}C)_4$ (**7**)	33.1	250.4	3.9×10^4	0.0063	2.3
PEG_{10000}–$(AD)_2$–$(AD)_4$–$(Ara\text{-}C)_8$ (**8**)	45.3	291.1	5.7×10^4	0.0043	1.83

4. Conclusions

With suitable chemical route, Ara-C was conjugated to PEG of different weight and shape. Conjugation was achieved by acylation at the N^4 of the pyrimidine ring. By gel-filtration purification of conjugates it was possible to reduce the presence of free drug below the 0.7%, with respect to the total drug amount. The drug release rate from the conjugates is pH dependent, but it is also influenced by the polymer structure and molecular weight. The polymer moiety has an influence on the pharmacokinetic profile, thus PEG–$(AD)_2$–$(AD)_4$–$(Ara\text{-}C)_8$ (**8**), PEG–(AD_2)–$(Ara\text{-}C)_4$ (**7**) and $mPEG2_{20000}$–Lys–Ara-C (**5**) present a prolonged blood residence time, which is due to mass and shape of PEG for the compound **5** and to mass of PEG and branching of the polymer moiety in the case of compound **7** and **8**. These last two compounds possess the advantage of higher loading, which is a critical aspect in macromolecular prodrugs. The study demonstrated also the advantage of computational analysis in choosing convenient starting products to avoid the steric hindrance entanglement, a common problem in polymer synthesis.

5. Experimental procedures

5.1. Materials

Ara-C, 5′-*O*-trityl–Ara-C and Ara-U were Sigma Chemical Company products. Linear mPEG-OH (MW 5, 10, and 20 kDa), linear PEG (MW 10 and 20 kDa) and branched monomethoxypoly(ethylene glycol) (mPEG2–Lys–COOH) (MW 10 and 20 kDa) were obtained from Shearwater Polymer Inc. (Huntsville, AL). 2,4,6-Trinitrobenzensulfonic acid (TNBS), *p*-nitrophenyl chloroformate, *N,N*-dicychlohexylcarbodiimid (DCC), *N*-hydroxysuccinimid (NHS) and L-2-aminoadipic acid were purchased from Aldrich Chemie (Steinheim-West-Germany). Dimethylformamide (DMF) and other organic solvents and salts of analytical grade were from Merck. Nle was a Fluka product. Recombinant human cytidine deaminase (specific activity 2.95 U/ml) was kindly supplied by Vincenzetti et al. (Department of Veterinary Science, University of Camerino, Italy) and its enzymatic activity was evaluated according to Vincenzetti et al. [20].

For pH titration, a Radiometer Autoburette ABU 80 (Copenhagen, Denmark) with TTT80 and Titrigraph REA 160 was used. UV–vis analyses were performed on Perkin Elmer Lamba-5 instrument. Proton NMR spectroscopy was performed with Bruker 300 MHz spectrometer. Gel-filtration chromatography was performed on a Pharmacia LKB system using a Sephadex LH-20 column. Reverse phase chromatography was performed with a Shimadzu analytical and semi-preparative HPLC system using a Vydac C_{18} column, with UV detector. TNBS was used as reagent for quantitative amino group determination following the procedure described by Snyder [21]. PEG content was evaluated colorimetrically using the iodine assay [22].

5.2. Computational methodologies

Calculations were performed on a Silicon Graphics Octane R12000 workstation. Simplified models of PEG–Ara-C conjugates were built using the "Builder" module of Molecular Operating Environment (MOE 2002.03) [23]. Initial structures were minimised using MMFF94 force field [23–28], implemented by MOE modelling package, until the rms value of Truncated Newton method (TN) [29] was <0.0001 kcal mol^{-1} $Å^{-1}$. Charges for the PEG–Ara-C conjugates derivatives were imported from the MMFF94 force field database.

To exhaustively explore the conformational space of PEG–Ara-C conjugate structures, we performed a "Stochastic Conformational Search" implemented by MOE. The Stochastic Conformational Search method generates conformations by randomly sampling local minima of the potential energy surface. This method is similar to the RIPS method described by Ferguson and Raber [30] which generates new molecular conformations by randomly perturbing the position of each coordinate of the atoms in the molecule by some small amount, typically less than 2 Å, followed by energy minimisation. Each conformer was minimised using MMFF94 force field until the rms value of TN was <0.0001 kcal mol^{-1} $Å^{-1}$. Charges for PEG–Ara-C conjugate derivatives were imported from the MMFF94 force field database. To model the effects of solvent (water) more directly, a set of electrostatic interaction corrections are used. MOE suite implemented a modified version of GB/SA contact function described by Still and co-authors [31]. These terms model the electrostatic contribution to the free energy of solvation in a continuum solvent model.

5.3. Chemistry of PEG–Ara-C compounds

5.3.1. Preparation of mPEG–Nle–Ara-C (*1, 2, 3*) and PEG–$(Nle)_2$–$(Ara\text{-}C)_2$ (*6*)

5.3.1.1. Activation of mono and di-hydroxyl terminated linear PEG. Monofunctional mPEG–(*p*-nitrophenyl carbonate) (5, 10 and 20 kDa MW) and bifunctional PEG–(*p*-nitrophenyl carbonate)$_2$ (MW 10 kDa) were prepared by the same chemical route. For simplicity only the synthesis of $mPEG_{5000}$–(*p*-nitrophenyl carbonate) (**9**) is here reported:

One gram (0.2 mmol) of monomethyl poly(ethylene glycol) (mPEG-OH, MW 5 kDa) were dissolved in 20 ml of toluene and dehydrated by water–toluene azeotropic distillation. Eight millilitre of anhydrous CH_2Cl_2 were added to the polymer solution, followed by 121 mg (0.6 mmol) of *p*-nitrophenyl chloroformate and 83.5 μl (0.6 mmol) of Et_3N, to give **9**. The mixture was stirred for 6 h at room temperature, and then the product was precipitated by dropping the reaction mixture into 300 ml of diethyl ether. The resulting white solid was purified by repeated dissolution in CH_2Cl_2 and precipitation into diethyl ether (yield: 0.95 g, 95%). The degree of activation, evaluated on the basis of *p*-nitrophenol release, was 96%.

*5.3.1.2. mPEG–Nle–COOH (**10**) and PEG–(Nle–COOH)$_2$.* Synthesis of compounds mPEG–Nle–COOH (MW 5, 10 and 20 kDa) and PEG–(Nle–COOH)$_2$ (MW 10 kDa) were carried out through the same method, here is reported the preparation of mPEG$_{5000}$–Nle–COOH (**10**):

136.4 mg (1.04 mmol) of Nle were dissolved in 10 ml of CH_3CN/H_2O (2:3) with 218.8 μl (1.57 mmol) Et_3N and 900 mg (0.174 mmol) of **9**. The mixture was maintained at room temperature, under constant stirring, for 12 h, then the solution was acidified to pH 3.0 and *p*-nitrophenol was extracted from reaction mixture with diethyl ether (4 × 5 ml). The product was repeatedly extracted from the aqueous solution with chloroform (5 × 50 ml). The organic phase was dried with Na_2SO_4, concentrated under vacuum to 5 ml, and added dropwise to 200 ml of diethyl ether. The product **10** was collected by filtration and dried under vacuum (yield: 830 mg, 92%). The degree of functionalisation, evaluated by NaOH 0.01 N titration of the carboxylic groups, was 95%. By matrix assisted laser desorption ionization (MALDI) mass spectroscopy a single peak, centred in *m/z* = 5120 Da, was detected (PEG-OH had a *m/z* = 4960 Da). The peak showed the polydispersivity typical of PEG.

*5.3.1.3. mPEG–Nle–COOSu (**11**) and PEG–(Nle–COOSu)$_2$.* Seven hundred and fifty milligram (0.147 mmol) of **10**, were dissolved in 10 ml of anhydrous CH_2Cl_2 and the solution was cooled to 0 °C. 50.7 mg (0.440 mmol) of NHS and 90.8 mg (0.440 mmol) of *N,N′*-dicyclohexylcarbodiimid were added under stirring. The mixture was allowed to warm to room temperature and let reacting for 12 h. Dicyclohexylurea was removed by filtration and the solution, concentrated under vacuum, was dropped into 200 ml of diethyl ether. The product **11** was dried under vacuum (yield: 690 mg; 92%). The degree of activation, evaluated on the basis of the amino group modification toward amino group of H–Gly-Gly–OH as reported for Snaider's assay [14], was 93%. This value was in agreement with C_4-HPLC evaluation of NHS, which was released after incubation of sample of **11** in NaOH 0.2 N.

*5.3.1.4. mPEG–Nle–Ara-C (**1, 2, 3**) or PEG–(Nle)$_2$–(Ara-C)$_2$ (**6**).* For simplicity only the synthesis of mPEG$_{5000}$–Nle–Ara-C (**1**) is here reported:

10.3 mg (0.037 mmol) of Ara-C were dissolved at room temperature in 5 ml of anhydrous pyridine and the solution was cooled at 0 °C. One hundred and forty seven milligram (0.028 mmol) of **11** were added to the solution. The mixture was warmed to room temperature and let stirring for 72 h. After evaporation of pyridine the product **1** was purified, from unbound Ara-C and the low molecular weight contaminants by gel filtration using Sephadex LH-20 column, with DMF as eluent (0.2 ml/min). The collected fractions were analysed by UV absorption at 272 nm and by iodine assay for Ara-C and PEG determination, respectively. The fractions containing the desired conjugate were collected and the solvent was evaporated. The residue was dissolved in dichloromethane (4 ml) and added dropwise to 200 ml of diethyl ether. The precipitate was dried under vacuum (yield: 109 mg, 75%). **1** was analysed by RP-HPLC chromatography (Vydac C_{18} column—4.6 × 150 mm, particle size 5 μm) using 2.5 mM phosphate buffer pH 7.0 as eluent A and acetonitrile as eluent B. The gradient used was the following: 10 min at 0% of B, then from 0% to 50% B in 10 min and the last 10 min at 50% B. The flow rate was 1 ml/min and the UV detector was settled at 272 nm.

As an example UV–vis spectra and RP-HPLC elution profile of **1** are reported in Figs. 3 and 4, respectively.

H^1 NMR of **1**: (DMSO, δ ppm) 0.84 (s, 3H, CH_3 Nle); 1.26 (m, 4H, $CH_2\beta$ and γ Nle); 1.56 (m, 1H, $CH_1\alpha$ Nle); 3.24 (3H, s, OCH_3 of PEG); 3.56–4.16 (O–CH_2–PEG + H2′, H3′, H4′, 2H5′ Ara-C); 5.1 (s, 1H 5′OH Ara-C); 5.46 (s, 2H, 2′OH + 3′OH Ara-C); 6.04 (d, 1H, H1′ Ara-C); 7.17 (d, 1H, H5 Ara-C); 7.65 (d, 1H, OCONH PEG–Nle); 8.07 (d, 1H, Ara-C H6); 10.9 (s, 1H, CONH Nle–Ara-C).

5.3.2. Alternative chemistry route for preparation of PEG–Nle–Ara-C using 5′-O-trityl–Ara-C

28.3 mg (0.050 mmol) of 5′-*O*-trityl–Ara-C were dissolved at room temperature in 5 ml of anhydrous pyridine and the solution was cooled at 0 °C. Two hundred milligram (0.038 mmol) of **11** were added to the solution. The mixture was warmed to room temperature and let stirring for 72 h. After evaporation of pyridine the product **1** was purified, from unbound 5′-*O*-trityl–Ara-C and the low molecular weight contaminants by gel filtration using Sephadex LH-20 column, with DMF as eluent (0.2 ml/min). The collected fractions were analysed by UV absorption at 272 nm and by iodine assay for 5′-*O*-trityl–Ara-C and PEG determination, respectively. The fractions containing the desired conjugate were collected and the solvent was evaporated. The residue was treated with 50% acetic acid (5 ml) at 90 °C for 15 min; the product PEG$_{5000}$–Nle–Ara-C was recovered by extraction with CH_2Cl_2 (6 × 60 ml). The organic phase was dried and concentrated to 5 ml and added dropwise to 200 ml of diethyl ether. The precipitate was dried under vacuum (yield: 158 mg, 79%). The product was characterised as above reported. As already found for product **1**, obtained by direct acylation, H^1 NMR spectra showed the amide proton as expected and no signal caused by esterification. HPLC analysis demonstrated the identity of the products obtained by the two procedures. This result suggested to carry out the conjugation reaction by direct acylation of Ara-C with the various OSu activated PEGs as follows.

*5.3.3. Preparation of mPEG2–Lys–Ara-C (**4, 5**)*

*5.3.3.1. mPEG2–Lys–COOSu (**12**).* Activation of mPEG 2_{10000}–Lys–COOH MW (**12**) is reported as an example:

Eight hundred milligram (0.08 mmol) of mPEG2_{10000}–Lys–COOH, were dissolved in 12 ml of anhydrous CH_2Cl_2 and the solution was cooled to 0 °C. 27.6 mg (0.240 mmol) of NHS and 49.5 mg (0.240 mmol) of *N,N′*-dicyclohexylcarbodiimid were added under stirring. The mixture was

allowed warm to room temperature and react for 12 h. Dicyclohexylurea was removed by filtration and the solution, concentrated under vacuum, was dropped into 250 ml of diethyl ether. The product **12** was dried under vacuum (yield: 750 mg; 94%). The degree of activation, evaluated on the basis of the amino group modification toward amino group of H–Gly-Gly–OH as reported for Snaider's assay [21], was 92%. This value was in agreement with C_4-HPLC evaluation of NHS, which was released after incubation of sample of **12** in NaOH 0.2 N.

*5.3.3.2. mPEG2–Lys–Ara-C (**4, 5**).* The reaction between branched PEG and Ara-C follows the same chemical route of $mPEG2_{10000}$–Lys–Ara-C (**4**), which is here reported:

6.4 mg (0.023 mmol) of Ara-C were dissolved at room temperature in 5 ml of anhydrous pyridine and the solution was cooled at 0 °C. One hundred and eighty milligram (0.018 mmol) of **12** were added to the solution. The mixture was warmed to room temperature and stirred for 72 h. The product **4** was purified, from reaction mixture, and characterised as reported above (yield: 150 mg; 83%).

H^1 NMR spectra of **4**: (DMSO, δ ppm) 1.35–1.5 (m, 4H, $CH_2\beta$ and γ Lys); 1.71 (m, 2H, CH_2 β Lys); 3.22 (s, 6H, OCH_3 PEG); 3.41–4.03 (CH_2 PEG + H2′, H3′, H4′, 2H5′ Ara-C + CHα Lys); 5.1 (s, 1H, Ara-C 5′-OH); 5.47 (s, 2H, Ara-C 2′OH + 3′OH); 6.04 (d, 1H, Ara-C H1′); 7.17 (bs, 2H, H5 Ara-C + OCONHα Lys–PEG); 7.65 (bs, 1H, OCONHε Lys–PEG); 8.05 (d, 1H, H6 Ara-C); 10.91 (bs, 1H, NHCO Lys–Ara-C).

Yield: 133 mg, 74%.

*5.3.4. Preparation of PEG–$(AD)_2$–$(Ara\text{-}C)_4$ (**7**)*

*5.3.4.1. PEG_{10000}–(p-nitrophenyl carbonate)$_2$ (**13**).* One gram (0.1 mmol) of bifunctional poly(ethylene glycol) (HO–PEG_{10000}–OH, 10 kDa MW) was dissolved in 20 ml of toluene and dehydrated by water–toluene azeotropic distillation. Eight millilitre of anhydrous CH_2Cl_2 were added to the polymer solution, followed by 121 mg (0.6 mmol) of *p*-nitrophenyl chloroformate and 83.6 μl (0.6 mmol) of Et_3N, to give PEG_{10000}–(*p*-nitrophenyl carbonate)$_2$ (**13**). After stirring of the solution for 6 h at room temperature, the product was precipitated by dropping the reaction mixture into 300 ml of diethyl ether. The resulting white solid (**13**) was purified by repeated dissolution in CH_2Cl_2 and precipitation into diethyl ether (yield: 0.95 g; 95%). The degree of activation, evaluated on the basis of *p*-nitrophenol release, was 97%.

*5.3.4.2. PEG–$(AD)_2$–$(COOH)_4$ (**14**).* 83.8 mg (0.52 mmol) of L-2-aminoadipic acid (AD) were dissolved in 10 ml of CH_3CN/H_2O (2:3) with 217 μl (1.56 mmol) Et_3N and 900 mg (0.087 mmol) of **13**. The mixture was maintained at room temperature, under constant stirring, for 12 h, the solution was acidified to pH 3.0 and the *p*-nitrophenol was extracted from reaction mixture with diethyl ether (4 × 5 ml). The product **14** was repeatedly extracted from the aqueous solution with chloroform (5 × 50 ml). The organic phase was dried with Na_2SO_4, concentrated under vacuum to 5 ml, and added dropwise to 200 ml of diethyl ether. **14** was collected by filtration and dried under vacuum (yield: 830 mg, 92%). The degree of functionalisation, evaluated by titration of the carboxylic groups with NaOH 0.01 N, was 95%. By (MALDI) mass spectroscopy a single peak of polydispers product, centred in *m/z* = 10178 Da (HO–PEG_{10000}–OH had a *m/z* = 9840 Da), was detected.

*5.3.4.3. PEG–$(AD)_2$–$(COOSu)_4$ (**15**).* Seven hundred and fifty milligram (0.072 mmol) of **14**, were dissolved in 10 ml of anhydrous CH_2Cl_2 and the solution was cooled to 0 °C. 24.9 mg (0.216 mmol) of NHS and 44.6 mg (0.216 mmol) of *N,N′*-dicyclohexylcarbodiimid were added under stirring. The mixture was allowed to warm to room temperature and to react for 12 h. Dicyclohexylurea was removed by filtration and the solution, concentrated under vacuum, was dropped into 200 ml of diethyl ether. The product obtained, PEG–AD_2–OSu_4 (**15**), was dried under vacuum (yield: 690 mg; 92%). The degree of activation, evaluated on the basis of the amino group modification of H–Gly-Gly–OH as reported for Snaider's assay [21], was 91%. This value was in agreement with C_4-HPLC evaluation of NHS, which was released after incubation of sample of **15** in NaOH 0.2 N.

*5.3.4.4. PEG–$(AD)_2$–$(Ara\text{-}C)_4$ (**7**).* 20.1 mg (0.072 mmol) of Ara-C were dissolved at room temperature in 5 ml of anhydrous pyridine and the solution was cooled at 0 °C. One hundred and fifty milligram (0.014 mmol) of **15** were added to the solution. The mixture was warmed to room temperature and stirred for 72 h. The product PEG–AD_2–Ara-C_4 (**7**) was purified and characterised as reported above.

H^1 NMR spectra **7**: (DMSO, δ ppm) 1.28 (t, 4H, $CH_2\gamma$ AD); 2.03 (m, 4H, $CH_2\beta$ AD); 2.30 (m, 4H, $CH_2\delta$ AD); 3.56–4.16 (O–CH_2 PEG + H2′, H3′, H4′, 2H5′ Ara-C); 4.25 (m, 2H, CHα AD); 5.1 (bs, 4H, 5′OH Ara-C); 5.46 (bs, 8H, 2′OH + 3′OH Ara-C); 6.04 (d, 4H, H1′ Ara-C); 7.17 (d, 4H, H5 Ara-C); 7.60 (bs, 2H, OCONH PEG–AD); 8.07 (d, 4H, H6 Ara-C); 10.9 (bs, 4H, CONH AD–Ara-C).

Yield: 119 mg, 79%.

*5.3.5. Preparation of PEG–$(AD)_2$–$(AD)_4$–$(Ara\text{-}C)_8$ (**8**)*

*5.3.5.1. PEG–$(AD)_2$–$(AD)_4$–$(COOH)_8$ (**16**).* 99.9 mg (0.62 mmol) of L-2-aminoadipic acid (AD), was dissolved in 10 ml of CH_3CN/H_2O (2:3) with 216 μl (1.55 mmol) of Et_3N. After cooling at 0 °C, 560 mg (0.052 mmol) of **15** were added. The mixture was maintained at room temperature, under constant stirring, for 12 h. CH_3CN was eliminated under vacuum and the aqueous solution was acidified to pH 3.0. The product **16** was extracted with chloroform (5 × 50 ml); the organic phase was dried with Na_2SO_4, concentrated under vacuum to 5 ml, and added dropwise into 200 ml of diethyl ether. The precipitated product was collected by

filtration and dried under vacuum (yield: 500 mg, 89%). The degree of functionalisation, evaluated as reported for **14**, was 91%. By (MALDI) mass spectroscopy single peak of polydispers product, centred in *m/z* = 10759 Da (HO–PEG_{10000}–OH had a *m/z* = 9840 Da), was detected.

*5.3.5.2. PEG–$(AD)_2$–$(AD)_4$–$(OSu)_8$ (**17**).* Four hundred and fifty milligram (0.039 mmol) of **16**, were dissolved into 10 ml of anhydrous CH_2Cl_2 and cooled to 0 °C. 109.4 mg (0.95 mmol) of NHS and 196 mg (0.95 mmol) of *N,N′*-dicyclohexyl-
carbodiimid were added under stirring. The mixture was allowed to warm to room temperature and let stirring for 12 h. Dicyclohexylurea was removed by filtration and the solution, concentrated under vacuum, was dropped into 200 ml of diethyl ether to precipitate the product, PEG–AD_2–AD_4–OSu_8 (**17**) that was dried by vacuum (yield: 390; 87%). The degree of activation, evaluated as reported for **15**, was 90%. This value was in agreement with C_4-HPLC evaluation of NHS, which was released after incubation of sample of **17** in NaOH 0.2 N.

*5.3.5.3. PEG–$(AD)_2$–$(AD)_4$–$(Ara\text{-}C)_8$ (**8**).* 37.8 mg (0.135 mmol) of Ara-C were dissolved at room temperature in 5 ml of anhydrous pyridine and the solution was cooled at 0 °C. One hundred and fifty milligram (0.013 mmol) of **17** were added to the solution. The mixture was warmed to room temperature and let stirring for 72 h. The product, PEG–AD_2–AD_4–$(Ara\text{-}C)_8$ (**8**), was purified and characterised as reported above.

H^1 NMR spectra **8**: (DMSO, δ ppm) 1.28 (bs, 12H, $CH_2\gamma$ AD); 2.03 (bs, 12H, $CH_2\beta$ AD); 2.30 (m, 12H, $CH_2\delta$ AD); 3.56–4.16 (–CH_2–PEG + H2′, H3′, H4′, 2H5′ Ara-C); 4.25 (m, 6H, CHα AD); 5.1 (bs, 8H, 5′-OH Ara-C); 5.46 (bs, 16H, 2′OH + 3′OH Ara-C); 6.04 (d, 8H, H1′ Ara-C); 7.17 (d, 8H, H5 Ara-C); 7.60 (bs, 2H, OCONH PEG–AD); 8.07 (d, 8H, H6 Ara-C); 10–11 (bs, CONH of AD–Ara-C).

Yield: 119 mg, 79%.

5.4. Ara-C content in PEG conjugates

The content of Ara-C bound to PEG was determined after hydrolysis of the conjugate in NaOH 1 N [7]. The procedure for PEG_{10000}–$(AD)_2$–$(Ara\text{-}C)_4$ is reported as an example: 5 mg ($4.3\cdot\times 10^{-4}$ mmol) of conjugate were dissolved in 5 ml of NaOH 1 N and heated in water bath at 40 °C for 30 min. Two hundred microlitre of solution were neutralised with HCl 1 N, diluted to 1 ml with 2.5 mM phosphate buffer (pH 7.0) and analysed by RP-HPLC using the above reported gradient. The same HPLC method was also applied to an aqueous PEG_{10000}–$(AD)_2$–$(Ara\text{-}C)_4$ (**7**) solution before hydrolysis for the determination of the free drug present in the product: a calibration curve was established for Ara-C and used for the determination of free and total Ara-C in PEG–Ara-C conjugates.

5.5. In vitro conjugate hydrolysis

The release of Ara-C from PEG–Ara-C conjugates was investigated incubating the compounds ($3.5\cdot\times 10^{-4}$ mmol of bound Ara-C/ml) at 37 °C for 8–10 days in 0.07 M phosphate buffer at different pH values, namely 8.0, 7.4 and 6. Hundred microlitre samples were collected and analysed by RP-HPLC at appropriate times to evaluate the amount of Ara-C.

5.6. Conjugates stability in plasma

The stability of the PEG–Ara-C conjugates in plasma was studied by incubation of the polymer drug ($7\cdot\times 10^{-4}$ mmol of bound Ara-C/ml) in 2 ml of mouse plasma at 37 °C for 25 h. At scheduled times samples of 100 μl were analysed by adding uracile (50 μl) as internal reference standard and acetonitrile (450 μl) for deproteinisation. The mixture was centrifuged for 3 min at 12,000 rpm and 500 μl of clear supernatant were freeze-dried. The residue was dissolved in 2.5 mM phosphate buffer pH 7.0 and the components were evaluated by RP-HPLC.

5.7. Ara-C conjugates resistance to cytidine deaminase

Solutions of Ara-C or PEG–Ara-C conjugates were prepared in Tris–HCl, 0.1 M, pH 7.5 at 0.3 mM concentration with respect to the drug content. Ten microlitre of recombinant human cytidine deaminase, dissolved in PBS 50 mM 10% glycerol, were added to 1 ml of each solution, preincubated for 10 min at 37°C.

At appropriate intervals, aliquots of the reaction mixture (60 μl) were collected, 30 μl of acetonitrile were added to quench the reaction and the solvent was removed by freeze-drying. The residues were dissolved in 100 μl of water and analysed by RP-HPLC with a C_{18} Vydac column (4.6 × 150 mm, particle size 5 μm). The following gradient of water (A) and acetonitrile (B), both containing 0.05% (v/v) CF_3COOH was used: 0–10 min 5% B; 20 min 50% B 30 min 50% B. The flow rate was 1 ml/min and the UV detector was settled at 272 nm. The relative peak area determined the concentration of Ara-C and Ara-U.

5.8. In vitro cytotoxic activity

Cytotoxicity of PEG–Ara-C conjugates was studied in cancer human HeLa cells by means of MTT test. Cells were harvested from exponential phase culture and plated in 96-well plates (5×10^3 cell/well). After 24 h from plating, cells were incubated with culture medium containing increasing concentrations of tested compounds (Ara-C and PEG–Ara-C conjugates). Twenty-four hours after drug addition, cells were treated for 4 h with MTT (80.5 mg ml^{-1}) followed by overnight treatment with SDS 10% in HCl 0.01 M. The optical density was measured at 570 nm using a Camberra Packard microplate reader.

5.9. Pharmacokinetic studies of Ara-C and PEG–Ara-C conjugates

Free or conjugated Ara-C samples were dissolved in 2.5 ml of phosphate buffer pH 7.4 (7×10^{-3} mmol/ml) and administered via tail vein to two groups of five female mice Swiss (25–28 g), under light anaesthesia. The injected dose corresponded to 7.5 mg/kg of Ara-C, this amount of Ara-C corresponds to that usually used in pharmacokinetics studies. Blood samples (100 μl) were taken at different times from mice' heart with a heparinized syringe under diethyl ether induced anaesthesia. The blood samples were centrifuged at 12,000 rpm for 3 min and 50 μl of plasma were taken. Fifty microlitre of uracile 0.15 mg/ml solution were added to the plasma solution as internal standard. The deproteinisation of this solution was obtained by adding 300 μl of acetonitrile. The resulting mixture was centrifuged for 3 min at 12,000 rpm and 300 μl of clear supernatant were freeze-dried.

In the case of the Ara-C, the residue was solubilised with 150 μl of 2.5 mM phosphate buffer pH 7.0 and analysed by RP-HPLC. For PEG–Ara-C, the samples were previously hydrolysed with NaOH 1 N (100 μl) at 40 °C for 30 min to release free Ara-C. After neutralisation with 2.5 mM H_3PO_4, the obtained solutions were analysed by RP-HPLC. Elution was performed as reported above.

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