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ORIGINAL ARTICLE

Inclusion complexes of a nucleotide analogue with hydroxypropyl-beta-cyclodextrin

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Abstract Bis(*t*butyl-*S*-acyl-2-thioethyl)-AraCMP (UA911) is a mononucleotide prodrug developed to overcome some of the cellular resistance to cytotoxic deoxynucleosides analogues. Its use for in vivo studies is limited due to its poor solubility in water. Thus, 2-hydroxypropyl-beta-cyclodextrin (HP-β-CD) was proposed to solubilize UA911 in water, in order to obtain concentrations needed for in vivo experiments. A molar ratio of HP-β-CD: UA911 of 3:1 was sufficient to obtain complete solubilization of the prodrug. The corresponding inclusion complex was characterized by differential scanning calorimetry and ^{1}H NMR spectroscopy study provided a definitive proof of the formation of the inclusion complex. The complex retained its cytotoxic activity as shown by in vitro cell survival assays on murine leukemia cells, and was evaluated in vivo. HP-β-CD is therefore suitable for the preparation of adequate solutions for the study of the antitumoral activity of nucleotide prodrugs such as UA911.

Keywords 2-Hydroxypropyl-beta-cyclodextrin · Inclusion complexes · Pronucleotide · In vivo · AraC · Deoxynucleoside analogues

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Introduction

Deoxynucleoside analogues are critical components of anticancer, antiviral and immunosuppressive therapy. They are antimetabolites, and inactive prodrugs that are dependent on intracellular phosphorylation into their pharmacologically active triphosphate forms [1]. Their intracellular activation depends on enzymes involved in the metabolism of endogenous nucleosides, and in particular deoxycytidine kinase which catalyses the rate-limiting step. The clinical use of these molecules is limited by the emergence of mechanisms of resistance such as decreased deoxycytidine kinase activity, rapid inactivation by cytidine deaminase, dephosphorylation by intracellular 5′-nucleotidases, a very low lipophilicity, and quick excretion. To overcome some of these limitations, several groups are developing different strategies including prodrugs [1, 2]. We have developed a mononucleotide prodrug of cytarabine (*t*Bu-SATE-Ara-CMP or UA911), bearing two *t*butyl-*S*-acyl-thioethyl (*t*Bu-SATE) groups, providing biolabile phosphate protection, capable of releasing intracellularly the monophosphorylated deoxynucleoside analogue. This molecule broadens the therapeutic spectrum of Ara-C towards kinase-deficient tumor cells [3, 4], protects Ara-C from deamination since the major substrate requirement of cytidine deaminase is a free 5′-hydroxyl group, and increases the lipophilicity of the parent Ara-C.

Several studies have aimed to ameliorate the biological and chemical stability of deoxynucleoside analogue prodrugs, as for example by the introduction into nanocapsules and nanospheres [5–7]. For some compounds, poor

solubility in water or other solvents compatible for animal studies limit the antitumoral evaluation in in vivo models. For UA911, the solubility profile was not suitable for such investigations. We therefore proposed to use cyclodextrins in order to modify the physicochemical properties of UA911 by the formation of inclusion complexes.

Natural cyclodextrins (CD) constitute a family of cyclic oligosaccharides comprising repetitive 6, 7, or 8 glucose units (α-, β-, γ-CD, respectively). The inside of the molecule forms a hydrophobic cavity, while the outer surface is hydrophilic, enabling it to act as a host for a wide variety of lipophilic drugs and components [8]. By complexation, cyclodextrins can increase the solubility, stability, and bioavailability of the guest molecule [9]. We therefore proposed to use 2-hydroxypropyl-β-CD (HP-β-CD), a derivative of natural β-CD, because of its higher water solubility and lower toxicity than the natural product.

In this paper, we present the preparation procedure and characterization of HP-β-CD:UA911 inclusion complex, as well as in vitro and in vivo evaluation of the biological activity of the inclusion complex on deoxynucleoside analogue-sensitive and -resistant murine leukemic cells.

Materials and methods

Materials

Methylthiazoletetrazolium (MTT), isopropanol, ethanol and HP-β-CD were purchased from Sigma Aldrich (Saint-Quentin, France), HCl from Merck (Strasbourg, France), RPMI 1640 cell culture media from Invitrogen (Cergy Pontoise, France), L-glutamine and penicillin-streptomycin from Gibco (Cergy Pontoise, France), and fetal bovine sera from PAN Biotech GmbH (Aidenbach, Germany). UA911 was obtained as previously described [10], and Ara-C (Aracytine®) was from Pharmacia (Saint-Quentin-en-Yvelines, France).

Preparation of inclusion complexes

1.5 mL of aqueous suspensions with different molar ratios (1:1, 2:1, 3:1, 4:1, 5:1) of HP-β-CD and UA911 were shaken at the temperature of 25 °C for 72 h to obtain an equilibrated state of solubilization.

For the in vitro cytotoxicity studies, two aqueous solutions (1.5 mL) of inclusion complex (HP-β-CD:UA911) with molar ratio about 3:1 were prepared at 10 or 60 mM of UA911.

Differential scanning calorimetry (DSC)

The characterization of HP-β-CD, UA911 and the inclusion complex (HP-β-CD:UA911) was carried out with DSC (TA instruments DSC 2920 Modulated DSC, New Castle, DE, USA). Each sample was scanned at a speed of 10 °C/min, in the temperature range of 20–250 °C.

Proton Nuclear Magnetic Resonance spectroscopy (^{1}H NMR)

The inclusion complex (HP-β-CD:UA911) was characterized in deutered water by ^{1}H NMR. The spectrum was obtained in a DRX 500 MHz Brucker spectrophotometer, at 298 K. Chemical shifts caused by complexation reaction between HP-β-CD and UA911 was measured in order to prove the inclusion of UA911 in the cyclodextrin cavity.

Cell culture

Deoxynucleoside analogue-sensitive (wt) and -resistant (10 K) L1210 murine leukemic cells were grown in RPMI 1640 media containing L-glutamine, penicillin (200 UI/mL), streptomycin (200 µg/mL) and fetal bovine serum (10%) at 37 °C in presence of CO_2 5%. The L1210 10 K cell line is described elsewhere [4].

Cytotoxicity studies

L1210 cells (20,000 cells per well) were incubated in 24 well plates (Becton Dickinson, NJ, USA) in a final volume of 1 mL containing different drug concentrations at 37 °C for 72 h. MTT (500 µg) was added and after 2 h of incubation at 37 °C, the supernatant was replaced with 300 µL isopropanol/HCl/H_20 (v/v/v 90/1/9) to solubilize the formazan crystals. Spectrophotometrical determination of optical density was performed using a microplate reader (Labsystem Multiskanner RC). The IC_{50} (inhibitory concentration 50%) was defined as the concentration inhibiting proliferation to a level equal to 50% of that of controls, and the resistance ratio was the ratio between the IC_{50} of the deoxynucleoside anlaogue-resistant L1210 10 K cell line and the IC_{50} of the sensitive parental cell line L1210 wt. IC_{50} values were determined from concentration-effect curves generated using Microsoft Excel. Statistical significance was determined using Student's *t*-test.

In vivo studies

On day 0, groups of 6-week-old female B6D2F1 mice (IFFA CREDO, Arbresle, France) received intraperitoneal injections containing 10^6 L1210 wt or L1210 10 K cells in exponential growth phase. Ara-C (75, 150 and 300 mg/kg) and UA911 in inclusion complex (187.5, 375 and 750 mg/kg) were administered intraperitoneally on days 1–4 and days 7–10 at equimolar doses of Ara-C and UA911. A group of control mice received injections of NaCl

(untreated), and another group received HP-β-CD similar to the dose used in the highest dose of inclusion complex (5375 mg/kg).

Results and discussion

Inclusion complex formation

UA911 is a water insoluble prodrug. Therefore, in order to increase its apparent aqueous solubility HP-β-CD was proposed. Suspensions with different molar ratios (1:1, 2:1, 3:1, 4:1, 5:1) of HP-β-CD and UA911 were shaken at the temperature of 25 °C. After 15 min, the preparations with molar ratios greater or equal to 3 became completely transparent, indicating a complete solubilization of UA911 by the formation of inclusion complexes with HP-β-CD. It was noticed that the formation of the inclusion complex was obtained in a very short time compared to periods usually needed for the preparation of inclusion complexes with cyclodextrins in aqueous solution (15 min vs. 3–11 days). The extreme rapidity of the formation of inclusion complexes could be justified by the short hydrophobic chains of methyl in the *ter*-butyl extremity of UA911, which can easily fill the hydrophobic cavities of HP-β-CD molecules. We decided to continue our study with an inclusion complex consisting of a molar ratio of 3:1 HP-β-CD:UA911.

Differential scanning calorimetry (DSC)

To verify the formation of inclusion complex (HP-β-CD:UA911), the pure products (HP-β-CD, UA911) and the inclusion complex were analyzed by DSC as shown by the thermograms depicted in Fig. 1. The active substance (UA911) showed a characteristic narrow peak at 168 °C corresponding to the melting point of the drug (Fig. 1a). Pure HP-β-CD had a broad peak which reached a maximum at approximately 90 °C (Fig. 1b). This phenomenon might be attributed to the loss of water molecules contained in the cavity of HP-β-CD inducing its dehydration. The thermal analysis of the complex (HP-β-CD:UA911) revealed the disappearance of the melting phenomenon described for the drug indicating the interaction of UA911 and the HP-β-CD cavity (Fig. 1c).

DSC analysis can be used to obtain information about the structural modifications in the internal cavity of cyclodextrin induced by the inclusion phenomenon. In fact, if this cavity contains only guest molecules, we should not have any dehydration peak in the DSC spectrum of the final mixture. However, experimental results generally show the persistence of this peak with a more or less significant modification of its characteristics. According to the literature, it is well known that if the guest molecule forms an inclusion complex with cyclodextrin, there is no crystalline structure to absorb energy, and then the formation of the inclusion complex can be confirmed by the disappearance of the endothermic melting peak in the complex thermogram.

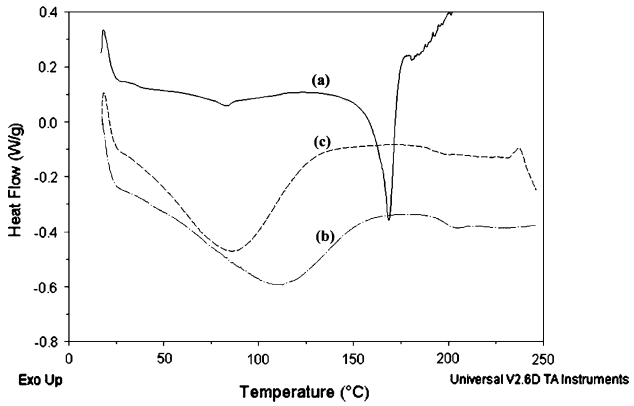


Fig. 1 DSC thermograms of: (**a**) UA911, (**b**) HP-β-CD and (**c**) inclusion complex (HP-β-CD:UA911)

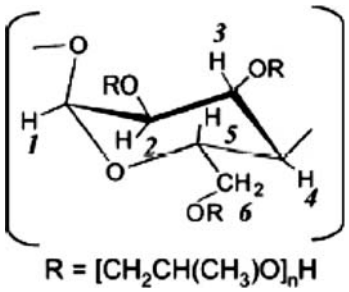
^{1}H NMR spectroscopy

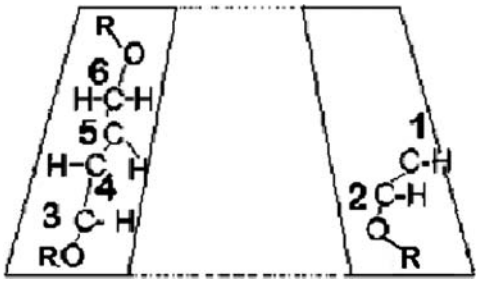
To provide a definitive proof of the formation of the inclusion complex, we realized ^{1}H NMR spectroscopy study, since the chemical and electronic environments of protons are affected during complexation, which is reflected by changes in the chemical shifts ($\Delta\delta = \delta_{(\text{complex})} - \delta_{(\text{free})}$).

Figure 1 presents the spectrum realized on the inclusion complex (HP-β-CD:UA911) and the pure HP-β-CD. For HP-β-CD in the presence of UA911, appreciable chemical shift displacements were observed with respect to the spectrum of the free compound, due to some conformational changes having occurred via the complexation. The protons H5, H3 and H6 located within or near the internal cavity were markedly affected with $\Delta\delta$ values of -0.070, -0.028 and -0.028 ppm, respectively (Table 1). The signals of H2 and H4 protons on the outer surface of HP-β-CD changed only slightly, whereas for the proton H1, the $\Delta\delta$ is -0.046. Therefore, we can suppose that a part of the UA911 molecule is included within the HP-β-CD cavity and the other part interacts with its outer surface and especially with the proton H1. Chemical shifts of protons other than those listed in Table 1 could not be accurately measured because of the overlapping and broadening of signals.

As reported in the literature for cyclodextrins, the displacements which were generally observed in the chemical shifts of the protons located within the internal cavity (H3, H5) because only this part is in contact with the guest molecule [11, 12]. In certain cases, chemical shifts

Table 1 ^{1}H NMR chemical shifts displacements (ppm) of HP-β-CD in D_2O at 298 K, in the free and complexed states at 3:1 molar ratio of HP-β-CD:UA911





HP-β-CD Protons	δ HP-β-CD	δ HP-β-CD:UA911	$\Delta\delta$[a]
H3	3.945	3.917	−0.028
H6	3.788	3.760	−0.028
H5	3.646	3.576	−0.070
H2	3.531	3.546	0.015
H4	3.409	3.403	−0.006
H1	5.184	5.138	−0.046
–CH3	[b]	[b]	[b]

[a] Chemical shift displacements were expressed as $\Delta\delta = \delta_{(\text{HP-}\beta\text{-CD})} - \delta_{(\text{HP-}\beta\text{-CD:UA911})}$

[b] Could not be determined due to the overlapping with other signals

displacements of external protons (H1, H2, and H4) can be observed. This occurs when the encapsulated molecule has a large size and when it presents one or more aliphatic chains which can remain outside the cavity and interact with the external faces of cyclodextrins [13].

In vitro cytotoxicity studies

In vitro cytotoxicity studies were performed using deoxynucleoside analogue sensitive (wt) and resistant (10 K) L1210 cells. In this study, we compared the cytotoxic effects of Ara-C, UA911 in DMSO and two concentrations of the inclusion complexes HP-β-CD:UA911 (10 mM and 60 mM) in MTT assays (Table 2). As we reported before [4], L1210 wt cells were 10-fold less sensitive to UA911 cytotoxicity than to Ara-C cytotoxicity (IC_{50} values of 0.0767 μM vs. 0.00767 μM, respectively), whereas L1210 10 K cells were 9-fold more sensitive to UA911 than to Ara-C (8.33 μM vs. 73.3 μM, respectively). The resistance ratios were 112 for UA911 and 9947 for Ara-C, respectively ($p = 0.018$). IC_{50} values and resistance ratios for the inclusion complex were not different from those obtained with UA911 diluted in DMSO, indicating that HP-β-CD did not modify the cytotoxicity of UA911 on L1210 wt and 10 K cells.

These results confirmed those previously obtained on the partial reversion of the resistance of L1210 10 K cells by UA911 [4]. We also showed that the presence of HP-β-CD did not alter the biological activity of the prodrug, and we were therefore successfully able to substitute the toxic organic solvent DMSO by a safe aqueous solution of the UA911, which can be used for in vivo administration.

In vivo studies of antitumor activity of the inclusion complex

The in vivo antitumor activity of the inclusion complex containing UA911 was studied in mice using our previously described model (Table 3) [4]. A preliminary assay in groups of 3 mice showed that the dose of 750 mg/kg of UA911 was highly toxic inducing rapid death of treated mice. Death due to toxicity could be distinguished from death due to disease, as the latter was associated with the development of ascitis. No toxicity or antitumor activity was observed in the group treated with HP-β-CD only. The in vivo Ara-C-resistance of L1210 10 K cells was confirmed by a median survival of 27 days for mice with L1210 wt cells and treated with Ara-C and 14 days for mice with L1210 10 K cells and treated with Ara-C.

In a second study, using doses of 375 and 187.5 mg/kg of UA911 in groups of 5 mice, we observed a similar toxicity of the 375 mg/kg dose as that observed at 750 mg/kg in mice bearing L1210 wt cells or L1210 10 K cells. However, in mice with L1210 wt cells, the UA911 dose of 187.5 mg/kg induced the same prolongation of survival as an equimolar dose of Ara-C (75 mg/kg) when compared to untreated

Table 2 IC_{50} values and resistance ratios in L1210 wt and L1210 10 K cells as determined by MTT assay

	IC50 (μM)		RR	*p t*
	L1210 wt	L1210 10 K		
Arac	0.00767 ± 0.00116	73.3 ± 25.2	9.947 ± 4.365	0.0072*
UA911	0.0767 ± 0.0208	8.33 ± 2.89	112 ± 48.9	0.0077*
HP-β-CD:UA911 10 mM	0.123 ± 0.0681	20.0 ± 10.0	180 ± 105	0.026*
HP-β-CD:UA911 60 mM	0.0633 ± 0.0473	11.7 ± 7.64	283 ± 202	0.058

Note. Data are mean values of four independent experiments

RR: is the resistance ratio which is calculated as IC_{50} for L1210 10 K/IC_{50} for L1210 wt

p t is the value of *p* calculated by Student's *t*-test

* Statistically significant at a level of $p < 0.05$

Table 3 Results from in vivo experiments with UA911 in inclusion complex with HP-β-CD

Cell line	Treatment	Dose (mg/kg)	Number of mice	Median days of survival	Prolonged survival (versus control group)	Toxic deaths
L1210 wt	NaCl		7	16		0/7
	HP-β-CD	2687.5	5	16	0	0/5
	Ara-C	300	3	27	12[a]	0/3
	HP-β-CD:UA911	750	3	12	-	2/3
	Ara-C	150	7	21	5[b]	0/7
	HP-β-CD:UA911	375	5	15	–	3/5
	Ara-C	75	7	20	4[b]	0/7
	HP-β-CD:UA911	187.5	5	20	4[b]	0/5
L1210 10 K	NaCl		7	13		0/7
	HP-β-CD	2687.5	5	14	0	0/5
	Ara-C	300	3	14	–	3/3
	HP-β-CD:UA911	750	3	11	–	3/3
	Ara-C	150	7	13	–	1/7
	HP-β-CD:UA911	375	5	12	–	4/5
	Ara-C	75	7	13	–	0/7
	HP-β-CD:UA911	187.5	5	13	–	0/5

[a] Median survival for control group in this study was 15 days

[b] These prolongations were statistically significant at $p < 0.05$ when compared to control mice

control mice (median survivals: 20 days, 20 days and 16 days, respectively, $p = 0.006$). This latter dose of UA911 was not toxic for mice bearing L1210 10 K cells, but did not induce any modification of survival (median survivals of 13 days for UA911, Ara-C and untreated controls).

Several comments can be made regarding the results of the in vivo administration of U911. The lack of conclusive results on the activity of UA911 on L1210 10 K cells in vivo, can be explained by a biological inactivity of the inclusion complex and UA911 itself. The similar results obtained with UA911 and Ara-C in our last in vivo study suggests that UA911 is degraded to Ara-C before it attains the tumor cells. Another explanation can be an inadequate treatment schedule. Indeed, due to the difficulties to obtain large quantities of UA911, we were limited in terms of treatment schemes, and we might therefore have used

inadequate treatment schedules for the identification of the ability of UA911 to revert resistance to Ara-C in vivo. Additionally, the partial reversion of Ara-C resistance with UA911 observed in vitro could be too small to be observed in our in vivo model. Finally it is important to note that mice serum physiologically contains a high level of esterase activity. Although we did not measure esterase activity in the peritoneal liquid of the mice in our experiments, it is possible that UA911, a phophotriester compound, was rapidly degraded in this animal model.

Conversely the fact that equimolar doses of Ara-C and UA911 did not induce the same level of toxicy (0% (0/7) death with Ara-C and 60% (3/5) with UA911) strongly suggests that UA911 was not simply catabolized by esterase or hydrolysis into Ara-C. This enhanced toxicity of UA911, the nature of which has not been documented in this study, may be due either to altered drug kinetics or cellular permeability. Additional studies comparing the pharmacokinetics and pharmacodynamics of Ara-C and UA911 are clearly warranted.

Conclusion

In the present study, we proposed the use of HP-β-CD to overcome the problem associated with the in vivo use of UA911, i.e. its poor water solubility, by the formation of the inclusion complex of HP-β-CD:UA911. Thereby, we were able to prepare UA911 aqueous solutions at adequate concentrations for in vivo UA911 activity studies. The formation of inclusion complexes was confirmed by the DSC and ^{1}H NMR findings. Furthermore, the cytotoxic activity of the prodrug in vitro was not altered by the presence of HP-β-CD. We can therefore suppose that the prodrug molecules in cell culture are released from the cyclodextrine hydrophobic cavity, and then diffuse through cellular membranes before reaching their site of action. These results provided a basis for the potential therapeutic application of co-formulation of UA911 and other pronucleotides with HP-β-CD in cancer therapy.

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