Eur. J. Med. Chem. 33 (1998) 809–816

Synthesis of new cotelomers derived from tris(hydroxymethyl) aminomethane bearing arabinofuranosylcytosine moieties. Preliminary results on their in vitro and in vivo antitumoral activities

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(Received 18 March 1998; accepted 13 May 1998)

Abstract – As an approach to the development of drug delivery systems, a new class of low macromolecular carriers called telomers bearing both antitumor agent such as arabinofuranosylcytosine (Ara-C) and galactose moieties were synthesized. These compounds were prepared by telomerization of tris(hydroxymethyl) acrylamidomethane (THAM) or monogalactosylated THAM and Ara-C polymerizable derivatives in the presence of transfer reagent such as alkanethiol or perfluoroalkanethiol. Their antitumor activities were assessed both in vitro and in vivo against a mouse cell line, murine B16 melanoma. The biological results show that the cytotoxic effect of Ara-C is preserved in vitro and in vivo when the drug is grafted to the telomeric structure. © Elsevier, Paris

tris / telomers / prodrugs / arabinofuranosylcytosine / antitumoral activity

1. Introduction

Arabinofuranosylcytosine (Ara-C), the clinically useful antimetabolite of cytidine, is one of the most important antitumor agents, especially used in the treatment of various leukaemias, carcinomas of the colon, breast and ovary [1]. As many inhibitors of DNA synthesis, the therapeutic use of Ara-C is largely hindered by its toxicity to normal dividing cells. Moreover, this drug is subject to rapid enzymatic deactivation by cytidine deaminase to arabinofuranosyluridine (Ara-U) [2]. Consequently, the patients plasma half-life of Ara-C is generally too short to provide an effective medication [3] and its use requires a very complex and precise dosage. Several modifications have been envisaged in order to minimize such drawbacks [1]. One of the possibilities for enhancing the pharmacological properties of this antitumor agent and/or to modify its pharmacokinetics and decrease its toxicity is to link it to a carrier. Indeed, grafting drug to natural or synthetic macromolecular carriers is expected to modulate several parameters including ability to cross membranes, absorption and elimination rates. Such modifications could afford a sustained activity and/or a drug latency [4, 5]. Furthermore, it is well known that specific recognition of carbohydrates by membrane lectins is involved in numerous biological events [6]. As a result, synthesis of macromolecules bearing pendant carbohydrates could insure a selective cell recognition and site-specific drug delivery.

With these objectives in mind and in an attempt to broaden the overall scope of macromolecules used as in vivo drug carriers, we designed a new class of low macromolecular delivery systems called telomers [7, 8]. These compounds were obtained by free radical telomerization of an acryloyl monomer such as tris(hydroxy-

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Abbreviations: AIBN, α,α′-azobisisobutyronitrile; Ara-C, Arabinofuranosylcytosine; β-Ala, β-Alanine; DCC, 1,3-dicyclohexylcarbodiimide; DCU, 1,3-dicyclohexylurea; DPn, number average degree of polymerization; Gaba, γ-amino butyric acid; THAM, tris(hydroxymethyl)acrylamidomethane; Tris, tris(hydroxymethyl) aminomethane

methyl)acrylamidomethane (THAM) **1**, in the presence of an alkane/fluoroalkanethiol as a transfer reagent [9]. Previous biological studies have shown that amphiphilic oligomers (named H-TAC or F-TAC) obtained in this way exhibit good biocompatibility, ubiquitous distribution in rat after i.v. or p.o. administration and display a long half-life (30–50 h) without any degradation in both plasma and tissues [10]. The bi-exponential elimination kinetics and the persistence of the activity can be explained in terms of a slow cellular exocytosis [10]. The cell uptake and cytoplasmic subcellular distribution of these telomers in two cell lines, malignant murine B16 melanoma and normal rat skin fibroblasts, have also been explored by Secondary Ion Mass Spectrometry [11]. These studies have clearly revealed the ability of F-TAC telomers to cross the plasma membrane and to distribute homogeneously within the cytoplasm of both normal and malignant cells. Furthermore, glycosylation of THAM hydroxyl functions could insure selective cell targeting *via* specific recognition of pendant carbohydrates by membrane lectins. In order to verify this assumption, the recognition ability of telomers derived from THAM bearing galactose moieties have been recently assessed and proved using the galactose specific KbCWL1 lectin (isolated from *Kluyveromyces bulgaricus* yeast) [12].

All of these results clarified the intracellular behavior of such compounds and appear to substantiate the fact that telomeric structures can be used as in vivo drug delivery systems, possibly to target tissues and organs.

In this paper, we describe the synthesis of cotelomers derived from THAM **1** or monogalactosylated THAM **2** (*figure 1*) and acrylamide monomer bearing arabinofuranosyl cytosine moieties grafted through a peptidic spacer arm such as β-alanine ($m = 2$) **6a** or γ-aminobutyric acid ($m = 3$) **6b**. These peptidic side chains should provide stability in the bloodstream of the drug grafting, but are likely to go through biodegradation by intracellular lysozomal enzymes [13]. We have focused our investigations on the in vitro and in vivo biological response of a mouse cell line, murine B16 melanoma, against the cytotoxicity of these cotelomers bearing Ara-C moieties and we tried to evaluate the importance of various chemical parameters such as the size of cotelomers, the presence of pendant galactose residues or the Ara-C level grafted on the macromolecule.

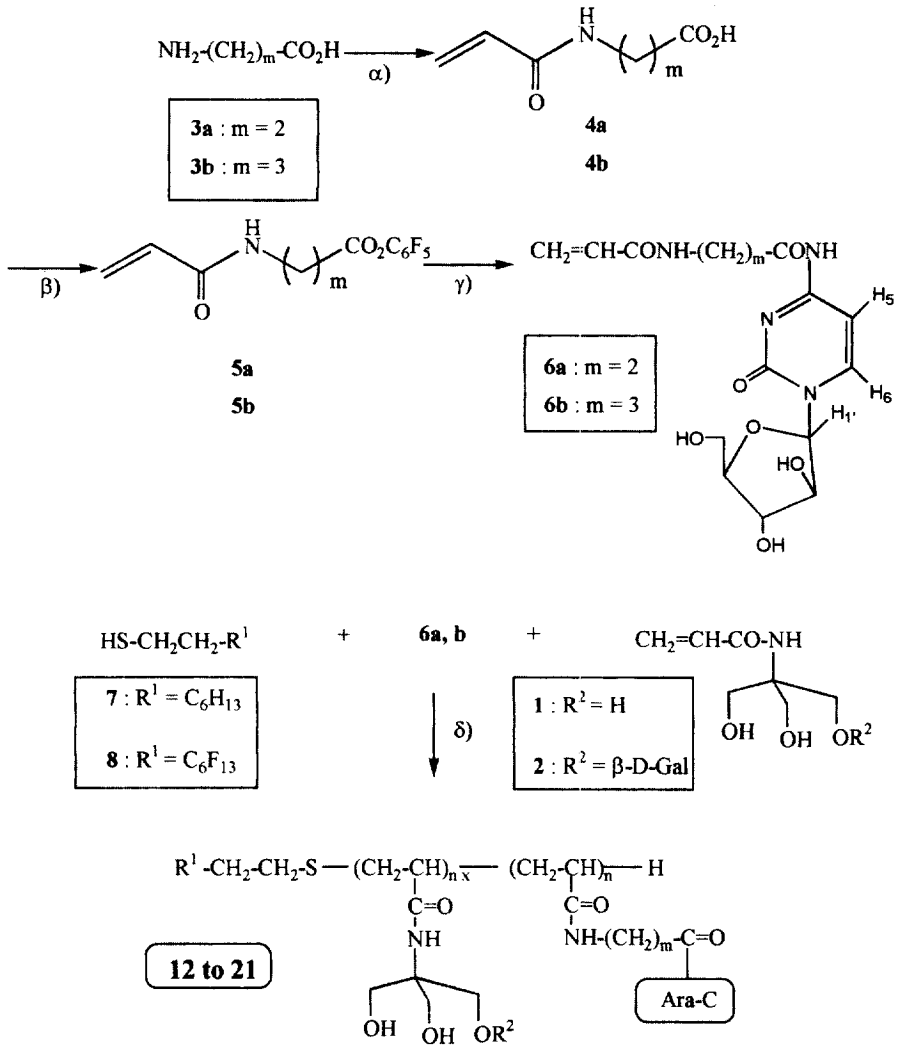
2. Chemistry

In previous reports, we described synthesis of tris(hydroxymethyl)acrylamidomethane (THAM) **1** and monogalactosylated THAM **2** [14, 15]. Telomerizations of such monomers in the presence of alkanethiol or perfluoroalkane thiol as transfer reagent provide different amphiphilic telomers [9, 10]. The multifunctional molecules obtained by this method exhibit physicochemical properties and biocompatibility making them suitable for drug delivery systems [8, 9]. In this way, we chose to use such macromolecular surfactants as drug carriers and we report herein the preparation of different cotelomers derived from THAM **1** or monogalactosylated THAM **2** and suitable Ara-C monomers (*figure 1*). The macromolecular prodrugs **12–21** (see *figure 1*) were conveniently prepared from pro-moieties **1** or **2** and **6a** or **6b** in the presence of 1-octanethiol **7** or 1H, 1H, 2H, 2H perfluorooctanethiol **8** as transfer reagent (or telogen) and a radical initiator (α,α′-azobisisobutyronitrile: AIBN).

Concerning preparation of monomers derived from Ara-C **6a** and **6b**, first of all a polymerizable function is introduced on the peptidic spacer **3a** or **3b** through acryloyl chloride. Condensation is realized in a methanolic solution of potassium hydroxide at 0 °C within a pH range between 8 and 9. Then, in order to link N-acrylamido peptidic acids **4a,b** to Ara-C hydrochloride in good conditions, their pentafluorophenol esters were previously synthesized. Consecutive coupling of these active esters **5a,b** to the drug were realized in pyridine. The Ara-C polymerizable monomers **6a,b** were then purified and isolated as white powders.

Telomerization experiments were performed in dimethylformamide at 80 °C under a nitrogen atmosphere, using AIBN as radical initiator. The AIBN concentration in the reaction mixture was roughly ten times lower than the telogen's [16]. The number average degree of polymerization (DPn) is equal to the amount of repeating units ($nx + n$). With a given transfer reagent, it may vary from one (monoadduct) to several tens, depending on the (telogen)/(monomer) ratio (R_0) adjusted through both starting material and experimental conditions [7]. The proportions of monomers **6a,b** and octanethiol **7** or 1H, 1H, 2H, 2H perfluorooctanethiol **8** (telogen) used are reported in *table I*. These proportions were chosen taking into account previous results obtained with THAM telomerization [9, 14]. Each experiment was pursued until the complete disappearance of the monomers. Telomers were purified by chromatography through a Sephadex G25 column and then lyophilized. The structures of these cotelomers, i.e. the relative proportions of each THAM (nx) or Ara-C (n) moiety in the cotelomer and the DPn of the macromolecule, were determined by elemental analysis and/or in ^{1}H-NMR by comparing the area of typical signals of each monomer.

For example, in the case of hydrocarbon telomers **11–15**, **18** and **21**, nx and n values were determined by comparing peaks area assigned to the terminal methyl



α) $CH_2CHCOCl$, CH_3OH, KOH 3N, 0 °C
β) C_6F_5OH, DCC, CH_2Cl_2, 20 °C
γ) Ara-C, DABCO, C_6H_5N, 80 °C, N_2
δ) AIBN, DMF, 80 °C
*each cotelomer have a statistical repartition of the different monomeric moieties

Figure 1. Synthesis of telomeric carriers derived from THAM.

signal in the hydrocarbon tail (δ 0.9 ppm, integral 3H) respectively to hydroxyl groups of THAM (δ 5 ppm, integral 3*nx*H) or monogalactosylated THAM (δ 5 ppm, integral 6*nx*H) and to H_1, proton of Ara-C moieties (δ 6.2 ppm, integral *n*H). Concerning fluorocarbon telomers **16**, **17**, **19** and **20** only the ratio *nx*/*n* was measured by ^1H NMR using the above process. However, the elemental analysis allowed us to estimate the term $(nx + 4n)$, then *nx* and *n* from the ratio %N/%F, according to the following equation:

$$\frac{\%N}{F} = \frac{14*(nx + 4n)}{13*19}.$$

Table I. Physico-chemical data and biological activity of cotelomers.

Compounds	R^1	R^2	R_0 [a]	m	nx [b]	n [b]	DPn [c]	Mw	IC_{50} [d]
Ara-C	–	–		–	–	–	–	243	0.039 ± 0.003
6b	–	–		3	–	–	–	383	0.19 ± 0.12
9	C_6H_{13}	–		2	–	1	1	514	0.245 ± 0.042
10	C_6F_{13}	–		3	–	1	1	762	0.37 ± 0.045
11	C_6H_{13}	–	10	2	–	12	12	4573	0.065 ± 0.010
12	C_6H_{13}	H	1/4	2	5.5	3.2	8.7	2286	0.021 ± 0.005
13	C_6H_{13}	H	5/8	2	8	1	9	1928	0.044 ± 0.008
14	C_6H_{13}	H	1/10	2	16	8	24	5988	0.046 ± 0.009
15	C_6H_{13}	H	1/30	2	50	25	75	18102	0.015 ± 0.004
16	C_6F_{13}	H	1/4	2	7.5	5.5	13	7700	0.072 ± 0.009
17	C_6F_{13}	H	5/8	2	8.5	1.1	9.6	2284	0.05 ± 0.004
18	C_6H_{13}	Gal [e]	1/4	2	5.5	3.6	9.1	3313	0.28 ± 0.02
19	C_6F_{13}	Gal [e]	3/8	3	5	1	6	2376	0.56 ± 0.063
20	C_6F_{13}	Gal [e]	5/8	3	8.4	1	9.4	3606	0.39 ± 0.14
21	C_6H_{13}	Gal [e]	1/15	2	60	32	92	32174	0.48 ± 0.032

[a] R_0 = $[Monomer]_0/[Telogen]_0$ is the ratio of the initial concentrations of the monomers and telogen. [b] nx and n are respectively the average number derived from THAM **1** or **2** and Ara-C **6a** or **6b** in the cotelomer. [c] DPn = number average degree of polymerization equal to $n(x + 1)$. [d] IC_{50} = concentrations of telomer (expressed in μM ± SD) giving a 50% efficiency compared to untreated melanoma cells. Each value is the average of three experiments. [e] Gal = β-D-galactopyranosyl.

3. Pharmacology

Cytotoxicity was tested both in vitro using the colony forming method on B16 cells, a mouse melanoma cell line, and in vivo on mice bearing P388 leukemia. In vitro results were expressed as IC_{50} (drug or prodrug concentration giving a 50% coloning efficiency compared to untreated cells). In vivo results were expressed as median survival times (MST), determined for the respective groups. Antitumor activity was measured by comparing the median survival time of the treated animals (T) with the one of controls (C) and was expressed as an oncostatic index: $T/C \times 100$ (see experimental section).

4. Results and discussion

As many antitumoral nucleoside analogues, the cytostatic effect of Ara-C depends on the kinase-mediated phosphorylation of its primary hydroxyl group, so that the drug can inhibit the DNA synthesis [3, 17]. Furthermore, it is worth noting that the inactivating enzyme cytidine deaminase induces a rapid metabolization of Ara-C into arabinosyluracil. Keeping in mind these natural occurrences, we synthesized new derivatives of Ara-C. In these compounds, the 4-amino group of cytidine was grafted to the α-carbon atom of a peptidic spacer. In our approach towards macromolecular prodrugs, such a peptidic spacer was used in order to introduce a polymerizable residue on the molecule without modifying the drug activity and furthermore to favour its liberation. Indeed, following the cell internalization of the conjugate, the cleavage of this amide bond by cytoplasmic peptidases could lead to the parent cytotoxic drug release.

Various targetable drug delivery systems have already been proposed using proteins, natural synthetic polymers or liposomes as drug carriers. In an attempt to show that grafting an antitumor agent (Ara-C) to low macromolecular structures could be a convenient chemotherapeutic approach, we focused our investigations on the design of several telomers derived from THAM or monogalactosylated THAM bearing Ara-C moieties.

A series of amphiphilic telomers reported in *table I* were then synthesized. Different structural parameters likely to enhance the potency of the prodrug towards tumoral tissues have been considered and their influence evaluated. Among them, we especially studied the effect of the hydrophilic–lipophilic balance on the prodrug biological activity. In this way, we successively modulated the length and the nature of the hydrophobic tail, and the number of hydrophilic THAM or monogalactosylated THAM pendants on the macromolecular backbone as well. The impact of both the multiplicity of Ara-C moities and the size of the telomer (DPn) was also specified.

Table I illustrates the cytotoxic effect of each compound expressed as IC_{50}. Previous reports deal with the presence of galactose-binding lectins on the outer surface of melanoma B16-type cells [6, 18]. These data prompted us to explore the ability of monogalactosyl ligands containing telomers for site-specific Ara-C delivery.

Thus, four telomers endowed with both galactose and Ara-C residues were tested with this aim. Results were reported in *table I.*

Compounds described in this table deserve several comments:

(1) In the case of the in vitro assays, modifications given to the Ara-C molecule do not cancel the cytotoxicity of the drug. Keeping in mind that all compounds are stable within a pH range from 4 to 10 in aqueous solution, only an enzymatic hydrolysis of the amide bond in the intracellular medium could lead to the drug release. Thus, as the Ara-C activity occurs in the nuclear compartment and that no peptidase are present in the culture medium, we can assume that the telomeric prodrugs are able to cross the cell membrane bilayer.

(2) It is noteworthy that, compared with the parent drug Ara-C, both the monomeric compounds **6a** or **6b** (which have roughly the same cytotoxicity) and the monoadducts (**9** and **10**) exhibit a loss of biological activity. According to the assumption that low molecular weight compounds diffuse through the cell membrane, one can assume that any modification of their hydrophilic–lipophilic balance could alter the membrane crossing. Polymerizable monomer **6b** or monoadducts **9** and **10** exhibit a higher hydrophobic character than Ara-C which can be correlated to their lack of activity. In the case of hydrocarbon monoadduct **9**, its lipophilic character may allow the cell-membrane insertion (or anchorage) and then delay, or eventually inhibit, its cellular internalization. Concerning the low activity obtained with the perfluorocarbon monoadduct **10**, the substitution of the hydrocarbon tail (compound **9**) by a perfluorocarbon one (compound **10**) involves a 1,5-fold decrease of the biological activity. Moreover, such a displacement introduces a fluorophilic character. One can suppose that this property could partially or completely inhibit the fusion with the phospholipidic cell membrane bilayer and preclude the diffusion process.

(3) On the contrary, the homotelomer derivative **11** exhibits no substantial loss of cytotoxicity compared to free Ara-C. Telomerization reaction, i.e. the multiplication of the chemotherapeutic ligand on the macromolecular backbone, could thus insure the recovery of the antitumoral activity. This observation implies that the biological activity of the prodrug cannot be directly correlated to the number of active moieties on the whole macromolecular backbone. So, it becomes necessary to only take into account the activity of the whole telomer structure and not the number of active pendants.

The enhancement of therapeutic potency noted for telomer **11**, compared with that observed for compounds **6b**, **9**, **10**, could be imputed to a difference in their cellular penetration. Indeed, if a fusion of the hydrophobic terminus of these last compounds with the phospholipidic cytoplasma membrane can be suggested, it seems likely that internalization of telomeric derivatives involves an endocytosis phenomenon. Furthermore, these results could support the elective intracellular cytoplasmic localization of this kind of telomers observed using both ion and fluorescence microscopy [11, 19].

(4) Fluoro or hydrocarbon cotelomers **12–17**, bearing various Ara-C/THAM ratio, show a cytotoxic activity almost comparable to the parent drug one. Furthermore, they seem to be slightly more active than the homotelomer substrate **11**. Presence of THAM moieties probably confers to the whole molecule, in particular to each Ara-C residue, a higher degree of freedom, essential for the enzymatic drug release. It is noteworthy that for compounds **13** and **17**, exhibiting equal size and equal number of therapeutic ligands, the nature of the hydrophobic tail does not affect their cytotoxicity. Surprisingly, if we consider the hydrocarbon cotelomers **12–15**, we can observe that the total number of drug residues does not significantly interact with their biological response. Many hypotheses involving cell penetration mechanisms or drug accessibility could be proposed, but, at this time, the results obtained with this kind of drug do not allow a valuable interpretation of this phenomenon.

(5) With respect to galactosylated derivatives **18–21**, we can note that the introduction of galactose pendants on the macromolecular backbone induces a 10-fold decrease of the cytotoxicity. These results may be correlated to the high affinity of such carbohydrates for outer cell surface specific receptors. This phenomenon may delay the internalization of the prodrug in the cytoplasmic compartment. Indeed, in previous works, we followed the recognition ability of glycosylated telomers by fluorescence microscopy. It appeared from this study that such compounds seemed to be blocked on the peripheral surface of the cells when galactose specific receptors were present [Contino C., Briot M., Coulon J., Polidori A., Bonaly R., Pucci B., submitted for publication].

(6) In vivo experiments were done with one of the more effective derivative **14** on B16 melanoma cells. LD_{50} measurements showed no mortality in mice for doses up to 1 g/kg, maximal administrable dose by intravenous way. For Ara-C, LD_{50} was 200 mg/kg. The oncostatic index was measured on P388 leukaemia grafted to C57Bl6 mice using isotoxic doses toward B16 melanoma cells: 200 mg/kg for **14** and 10 mg/kg for Ara-C. Obtained values were 155% for Ara-C and 160% for **14**. These results clearly show that the cytotoxic effect of Ara-C is maintained in vivo when this drug is linked to a telomeric structure. On the other hand, general toxicity is greatly decreased for the telomer compared with the free drug.

5. Experimental protocols

The progress of the reactions and the homogeneity of the compounds were monitored by thin-layer chromatography (TLC, Merck F_{254}). Detection was achieved by either exposure of plates to UV light (254 nm) or by charring with a methanol–sulfuric acid (1:1) solution. Purifications were performed by column chroma-

tography over silica gel (Merck 60), or by gel-permeation on Sephadex G15 (Pharmacia LKB). Melting points were measured on an electrothermal 9100 type-apparatus and are reported uncorrected. The ^{1}H, ^{13}C and ^{19}F nuclear magnetic resonance (NMR) spectra were recorded at 250 MHz on a Bruker AC250 spectrometer in chloroform-d_3 ($CDCl_3$), methanol-d_4 (CD_3OD) or dimethyl sulfoxyde-d_6 (DMSO). The chemical shifts are expressed in parts per million (ppm) relative to tetramethylsilane as an internal reference for the ^{1}H and ^{13}C spectra. For the ^{19}F NMR spectra, the internal reference is $CFCl_3$. Optical rotations were measured with a Perkin-Elmer Model 241 polarimeter. Ultrasonication was performed with a Vibra cell sonicator (13 mm probe, 50% pulsed, power 7, room temperature). Elemental analyses were conducted by the Service Central de Microanalyse du CNRS at Montpellier (France). Analyses indicated by the symbols of the elements or functions were within ±0.4% of theoretical values.

Reactions were performed in anhydrous conditions under dry nitrogen. All the solvents were distilled and dried according to standard procedures. For telomerization step, the solutions were carefully deoxygenated by nitrogen bubbling before use. AIBN was purified twice by recrystallization from absolute ethanol.

THAM **1** and monogalactosylated THAM **2** were synthesized as described by Pucci et al. [14, 15].

5.1. Synthesis of monomers derived from ARA-C: example

*5.1.1. N^4-((N^β-acryloyl)propylamido)-1-β-D-arabinofuranosylcytosine **6a***

*N^β-Acrylamidopropionic acid **4a***: To a stirred solution of β-Alanine (5 g, 5.61 mmol) in methanolic potassium hydroxide 3 N, at 0 °C within a pH range between 8 and 9, acryloyl chloride (8.2 mL, 101 mmol) was added dropwise. The reaction mixture was stirred at 0 °C for 1 h and then allowed to come to room temperature. After 4 h, at room temperature, the pH of the solution was decreased to 4 with formic acid. The reaction mixture was then filtered and the filtrate evaporated in vacuo to a residue which was crystallized with ethyl ether to give **4a** (8 g, 99%): m.p. 70–71 °C; ^{1}H NMR (DMSO-d_6) δ 8.17 (t, 1H, NH), 6.29 (dd, 1H, vinylic CH), 6.09 (dd, 1H, vinylic CH_2), 5.57 (dd, 1H, vinylic CH_2), 3.3 (q, 2H, β-CH_2), 2.26 (m, 2H, α-CH_2); ^{13}C NMR (DMSO-d_6) δ 173.87(CO_2H), 164.36 (CONH), 131.95 (vinylic CH_2), 124.60 (vinylic CH), 35.69 (β-CH_2), 35.53 (α-CH_2).

*N^γ-Acrylamidobutyric acid **4b***: This compound was prepared from γ-aminobutyric acid and acryloyl chloride similarly to **4a** (85%): m.p. 91–92 °C; ^{1}H NMR (DMSO-d_6) δ 8.12 (t, 1H, NH), 6.26 (dd, 1H, vinylic CH), 6.10 (dd, 1H, vinylic CH_2), 5.60 (dd, 1H, vinylic CH_2), 3.15 (q, 2H, γ-CH_2), 2.24 (t, 2H, α-CH_2), 1.66 (m, 2H, β-CH_2); ^{13}C NMR (DMSO-d_6) δ 174.09 (CO_2H), 164.56 (CONH), 131.77 (vinylic CH_2), 124.85 (vinylic CH), 37.91 (γ-CH_2), 31.04 (α-CH_2), 24.49 (β-CH_2).

*N-β-Acrylamidopropionic pentafluorophenyl ester **5a***: Pentafluorophenol (6.81 g, 37.06 mmol) was added to a solution of **4a** (5 g, 34.9 mmol) and DCC (7.21g, 34.9 mmol) in dry ethyl acetate. The mixture was stirred for 15 h at room temperature and the precipitated DCU was filtered. The filtrate was evaporated under vacuum and the residue crystallized with ethyl acetate/hexane to give **5a** (7.67 g, 71%): m.p. 74.2–75.5 °C; ^{1}H NMR ($CDCl_3$) δ 6.35 (dd, 1H, vinylic CH), 6.15 (m, 2H, vinylic CH_2, NH), 5.72 (dd, 1H, vinylic CH_2), 3.74 (t, 2H, β-CH_2), 3.03 (t, 2H, α-CH_2); ^{13}C NMR ($CDCl_3$) δ 171.4 (CO_2), 166.52 (CONH), 137.8 (vinylic CH_2), 127.42 (vinylic CH), 41.33 and 31.65 (α and β-CH_2); ^{19}F NMR ($CDCl_3$) δ −122.01 to −131.42 (5CF).

*N-γ-Acrylamidobutyric pentafluorophenyl ester **5b***: This compound was prepared from **4b** similarly to **5a** in 80% yield; m.p. 79.5–80 °C; ^{1}H NMR ($CDCl_3$) δ 6.38 (dd, 1H, vinylic CH), 6.20 (dd, 1H, vinylic CH_2), 6.06 (t, 1H, NH), 5.73 (dd, 1H, vinylic CH_2), 3.52 (q, 2H, γ-CH_2), 2.79 (t, 2H, α-CH_2), 2.07 (m, 2H, β-CH_2); ^{13}C NMR ($CDCl_3$) δ 170.75 (CO_2), 167.41 (CONH), 139.01 (vinylic CH_2), 128.21 (vinylic CH), 40.12, 32.3 and 26.21 (γ,α and β-CH_2); ^{19}F NMR ($CDCl_3$) δ −122.3 to −132.5 (5CF).

*N-(2-(N^4-carbamoyl-1-β-D-arabino-furanosylcytosine)-ethyl acrylamide **6a***: 1,4-Diazabicyclo [2.2.2] octane (0.2 g, 1.78 mmol) was added in parts to a stirred suspension of arabinofuranosylcytosine hydrochloride (1 g, 3.57 mmol) in dry pyridine (50 mL). After 30 min, at room temperature and under a nitrogen atmosphere, **5a** (1.32 g, 4.29 mmol) was added and the reaction mixture was then stirred overnight at 80 °C. Solvent was evaporated under vacuum and the crude product purified by column chromatography on silica gel using ethyl acetate–methanol (80:20, v/v) as eluent, to afford the pure product **6a** as a white powder (1.25 g, 95%): m.p. 190–191 °C; $[\alpha]_D$ + 91.3° (*c* 1, DMSO); ^{1}H NMR (CD_3OD) δ 8.28 (d, 1H, H-6), 7.43 (d, 1H, H-5), 6.22 (m, 3H, vinylic CH_2, vinylic CH, H′-1), 5.66 (dd, 1H, vinylic CH_2), 4.28 (t, 1H, H′-2), 4.10 (t, 1H, H′-3), 4.03 (m, 1H, H′-4), 3.81 (dd, 2H, H′-5), 3.57 (t, 2H, β-CH_2), 2.72 (t, 2H, α-CH_2); ^{13}C NMR (CD_3OD) δ 173.56 (CONH), 168.29 (C-4), 164.11 (acrylic CO), 157.91 (C-2), 147.87 (C-6), 131.93 (vinylic CH_2), 126.83 (vinylic CH), 97.13 (C-5), 89.49 (C′-1), 87.41 (C′-4), 78.16 (C′-3), 76.53 (C′-2), 62.79 (C′-5), 37.65 (β-CH_2), 36.16 (α-CH_2). Anal. $C_{15}H_{20}N_4O_7$ (C, H, N, O).

*5.1.2. N-(3-(N^4-carbamoyl-1-β-D-arabino-furanosylcytosine)-propyl acrylamide **6b***

This compound was prepared from **5b** as for **6a** in 78% yield and had m.p. 187.8–188.8 °C; $[\alpha]_D$ + 96.1 (*c* 1, DMSO); ^{1}H NMR (CD_3OD) δ 8.25 (d, 1H, H-6), 7.44 (d, 1H, H-5), 6.21 (m, 3H, H′-1, vinylic CH, vinylic CH_2), 5.66 (dd, 1H, vinylic CH_2), 4.27 (t, 1H, H′-2), 4.09 (t, 1H, H′-3), 4.02 (m, 1H, H′-4), 3.83 (dd, 1H, H′-5), 2.50 (t, 2H, α-CH_2), 1.88 (m, 2H, β-CH_2); ^{13}C NMR (DMSO-d_6) δ 173.74 (CONH), 165.15 (C-4), 162.35 (acrylic CO), 154.86 (C-2), 146.91 (C-6), 131.81 (vinylic CH_2), 125.46 (vinylic CH), 94.72 (C-5), 87.21 (C′-1), 85.90 (C′-4), 76.33 (C′-3), 74.79 (C′-2), 61.20 (C′-5), 34.05 (γ-CH_2), 31.15 (α-CH_2), 24.58 (β-CH_2). Anal. $C_{16}H_{22}N_4O_7$ (C, H, N, O).

*5.2. Synthesis of monoadducts **9**, **10**: example*

*5.2.1. 3-octylsulfamyl-N-(2-N^4-carbamoyl-1-β-D-arabino-furanosylcytosine)-ethyl propanamide **9***

Monomeric substrate **6a** (0.165 g, 0.44 mmol), octanethiol (0.196 g, 1.34 mmol) and AIBN (0.080 g, 0.48 mmol) were dissolved in dry DMF and refluxed under nitrogen atmosphere for 3 h (until all the starting monomer was reacted as indicated by TLC silica gel; 20% MeOH in EtOAc). The solution was then concentrated under reduce pressure and the residue purified by column chromatography on silica gel (eluent EtOAc) to give **9** (0.151 g, 66%): m.p. 125–126 °C; ^{1}H NMR (CD_3OD) δ 8.26 (m, 2H, H-6, NH β-alanyl), 7.46 (d, 1H, H-5), 6.20 (d, 1H, H′-1), 4.26 (t, 1H, H′-2), 4.09 (m, 1H, H′-4), 3.81 (dd, 2H, H′-5), 3.49 (m, 2H, β-CH_2), 2.73 (m, 4H, CH_2S, CH_2CO), 2.43 (m, 4H, SCH_2, α-CH_2), 1.54 (m, 2H, CH_2CH_2S), 1.28 (m, 10H, $(CH_2)_5$), 0.89 (t, 3H, CH_3); ^{13}C NMR (CD_3OD) δ 174.47 and 173.62 (2 CONH), 164.12 (C-4), 157.88 (C-2), 147.81 (C-6), 97.11 (C-5), 89.44 (C′-1), 87.34 (C′-4), 78.10 (C′-3), 76.45 (C′-2), 62.73 (C′-5), 37.70 (α-carbonyl CH_2), 37.31 (β-CH_2), 36.12 (α-CH_2), 32.96–28.70 (n CH_2), 23.68 (CH_2S), 14.43 (CH_3). Anal. $C_{23}H_{38}N_4SO_7$ (C, H, N, O, S).

*5.2.2. 3-(2-perfluorohexyl)-ethylsulfamyl-N-(3-N^4-carbamoyl-1-β-D-arabino-furanosylcytosine)-propyl propanamide **10***

This compound was prepared from **6b** (0.150 g, 0.39 mmol), 1H, 1H, 2H, 2H perfluorooctanethiol (0.446 g, 1.17 mmol) and AIBN (0.070 g, 0.43 mmol) similarly to **9**, in 60% yield and had m.p. 110 °C (decomposition); ^{1}H NMR (CD_3OD) δ 8.26 (m, 2H, H-6,

Table II. Experimental data of cotelomer synthesis.

Compounds	R^1	R^2	m	R_0 [a]	Monomers	R_a [b]	Yield (%)
12	C_6H_{13}	H	2	1/4	**1–6a**	1/1	57
13	C_6H_{13}	H	2	5/8	**1–6a**	4/1	83
14	C_6H_{13}	H	2	1/10	**1–6a**	1/1	55
15	C_6H_{13}	H	2	1/30	**1–6a**	1/1	63
16	C_6F_{13}	H	2	1/4	**1–6a**	1/1	47
17	C_6F_{13}	H	2	5/8	**1–6a**	4/1	50
18	C_6H_{13}	Gal [c]	2	1/4	**2–6a**	1/1	80
19	C_6F_{13}	Gal [c]	3	3/8	**2–6b**	2/1	40
20	C_6F_{13}	Gal [c]	3	5/8	**2–6b**	4/1	60
21	C_6H_{13}	Gal [c]	2	1/15	**2–6a**	1/1	50

[a] R_0 = [Monomer]$_0$/[Telogen]$_0$ is the ratio of the initial concentrations of the monomers and telogen. [b] R_a = [THAM monomer **1** or **2**]$_0$/[Monomer **6a** or **6b**]$_0$. [c] Gal = β-D-galactopyranosyl.

NH gaba), 7.45 (d, 1H, H-5), 6.20 (d, 1H, H'-1), 4.26 (t, 1H, H'-2), 4.09 (t, 1H, H'-3), 4.01 (m, 1H, H'-4), 3.82 (dd, 2H, H'-5), 3.52 (m, 2H, γ-CH_2), 2.90–2.70 (m, 6H, CH_2CF_2, CH_2SCH_2), 2.45 (m, 4H, α-carbonyl CH_2, α-CH_2), 1.87 (m, 2H, β-CH_2); ^{13}C NMR (CD_3OD) δ 174.07, 173.56 (2 CONH), 164.07 (C-4), 157.87 (C-2), 147.81 (C-6), 97.06 (C-5), 89.46 (C'-1), 87.39 (C'-4), 78.14 (C'-3), 76.48 (C'-2), 62.74 (C'-5), 34.06 (γ-CH_2), 32.97 (CH_2-α CF_2), 31.16 (α-CH_2), 28.67 (β-CH_2), 23.36 (CH_2S); ^{19}F NMR (CD_3OD) δ –81.24 (3F, CF_3), –114.21 (2F, CF_2-α CH_2), –121.79 (2F, CF_2-β CH_2), –123.21 (2F, CF_2-γ CH_2), –123.85 (2F, CF_2-δ CH_2), –126.23 (2F, CF_2-ε CH_2). Anal. $C_{24}H_{27}F_{13}N_4SO_7$ (C, H, F, N, O, S).

5.3. Synthesis of telomeric substrates 11–21

5.3.1. Homotelomerization of 6a (11)

Monomer **6a** (0.2 g, 0.54 mmol) was dissolved in refluxing dry DMF, whereupon a solution of octanethiol (0.008 g, 0.054 mmol, R_0 = 10) in anhydrous DMF (2 mL) was added, a solution of AIBN (0.0045 g, 0.027 mmol) in dry DMF (1.12 mL) was then added and the mixture refluxed for at least 12 h until the complete disappearance of **6a** (monitored by TLC and detected to UV light). At about the middle of the reaction, further addition of AIBN (0.0045 g, 1.12 mL of the solution in DMF) was effected. When all the starting monomer was reacted, the solution was concentrated under vacuum and poured into diethyl ether. The precipitate obtained was filtered, thoroughly washed with diethyl ether, and dissolved in a few mL of water. The mixture was fractionated with a Sephadex G25 column (1.5 × 100 cm, eluent: H_2O) and the desired fractions (detected on TLC by charring with a methanol–sulfuric acid solution) were lyophilized to give a set of telomers **11** as a white powder (0.105 g, 50%). The DPn (12) was obtained by ^1H-NMR by comparing the area of the signal due to the methyl group (distinct triplet; δ 0.9) with the area of the signal ascribed to proton H'-1 (doublet; δ 6.2) of Ara-C residues.

5.3.2. Synthesis of cotelomers 12–21

General procedure: The same procedure as used for the preparation of homotelomer **11** was applied to the synthesis of cotelomers (**12–21**). Monomers **6a** or **6b** and THAM **1** or monogalactosylated THAM **2** were reacted with different amounts of alkanethiol (**7**) or perfluoroalkanethiol (**8**) telogen (R_0 = [Monomer]/[Telogen]) as indicated in *table I* in the presence of AIBN ([AIBN] = [Telogen]/2). The different ratio of THAM **1** or monogalactosylated THAM **2**/monomer **6a** or **6b** used for the synthesis of cotelomers **12–21** are indicated with the yield of reactions in *table II*. The DPn of hydrocarbon cotelomers (**12**, **13**, **14**, **15**, **18**, **21**) were estimated by ^1H-NMR by comparison of the area of the terminal methyl signal (0.9 ppm) with that of H'1 Ara-C (6.2 ppm) residues and hydroxyl groups of THAM or monogalactosylated THAM (near 5 ppm). Concerning the perfluorocarbon cotelomers (**16**, **17**, **19**, **20**), the DPn was estimated by elemental analysis.

5.4. Pharmacological assays

5.4.1. Cytotoxicity assay

Cytotoxicity assay was performed using the colony forming method on B16, a mouse melanoma cell line derived from spontaneous skin tumor in C57Bl6 mouse (ICIG, Villejuif, France). Cells were cultured as monolayers in 25 cm^2 culture flasks in Eagle's minimum essential medium (Gibco, Paisley, Scotland) supplemented with 10% foetal calf serum (Sigma). For the cytotoxicity assay, B16 cells were plated into 60 mm Petri dishes (200 cells/dish) and allowed to adhere for 20 h before treatment. After this time, medium was removed and replaced by new medium containing increasing drug concentrations. Incubation was conducted for 12 days at 37 °C in a CO_2 incubator. After this time, all dishes were rinsed with phosphate buffered saline 0.05 M pH 7.4, cells were fixed with methanol and stained with 0.2% crystal violet solution, and colonies (>50 cells) were counted.

The anti-proliferative activity was expressed as IC_{50} (inhibiting concentration 50%), the drug concentration giving a 50% coloning efficiency compared to untreated cells.

5.4.2. Toxicity assessment

OF1 female mice 5–7 weeks (Iffa-Credo, l'Arbresle, France) were randomly assigned to five groups of six mice (20–25 g) for each compound. Both drugs were dissolved into sterile NaCl 0.9%. Doses of Ara-C ranged from 50 to 500 mg/kg and doses of telomer **14** from 200 to 1000 mg/kg. Mice were weighed daily; when animals had loss of weight more than 25% or when they were expected to become moribund, according to guidelines of UKCCCR [UKCCCR committee, 1988], they were sacrificed by decapitation. LD_{50} were calculated using the simplified method of Behrens and Kärber [20].

5.4.3. Evaluation of antitumor activity

Murine P388 leukemia, obtained from ICIG (Villejuif, France), was maintained by weekly transplantation of the tumor cells into

the peritoneal cavity of male DBA/2 mice (IFFA CREDO, l'Arbresle, France). For antitumor test, 1×10^6 cells were intraperitoneally injected into male C57Bl6 mice IFFA CREDO (l'Arbresle, France). Each treated group consisted of six and control group of twelve mice. Drugs dissolved into NaCl 0.9% were administered intraperitoneally on days 1, 5 and 9. Control animals received only NaCl 0.9%. Median survival times (MST) were determined for the respective groups. Antitumor activity was determined by comparing the median survival time of the treated animals (T) with the one of controls (C) and was expressed as an oncostatic index: $T/C \times 100$.

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