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Synthesis of Cotelomers Derived from Tris(Hydroxymethyl)acrylamidomethane (THAM) Bearing Cytosine Arabinoside Moieties. Preliminary Investigation of their Biological Activity.

Christiane Contino[a], Monique Ollier[b], Jean Claude Maurizis[b], Jean Michel Lacombe[a] and Bernard Pucci[a*]

a) Université d'Avignon, Laboratoire de Chimie Bioorganique et des Systèmes Moléculaires Vectoriels, 33, rue Louis Pasteur, 84000 Avignon, France
b) Unité INSERM U71, Rue Montalembert, BP 184, 63005 Clermont Ferrand Cedex, France.

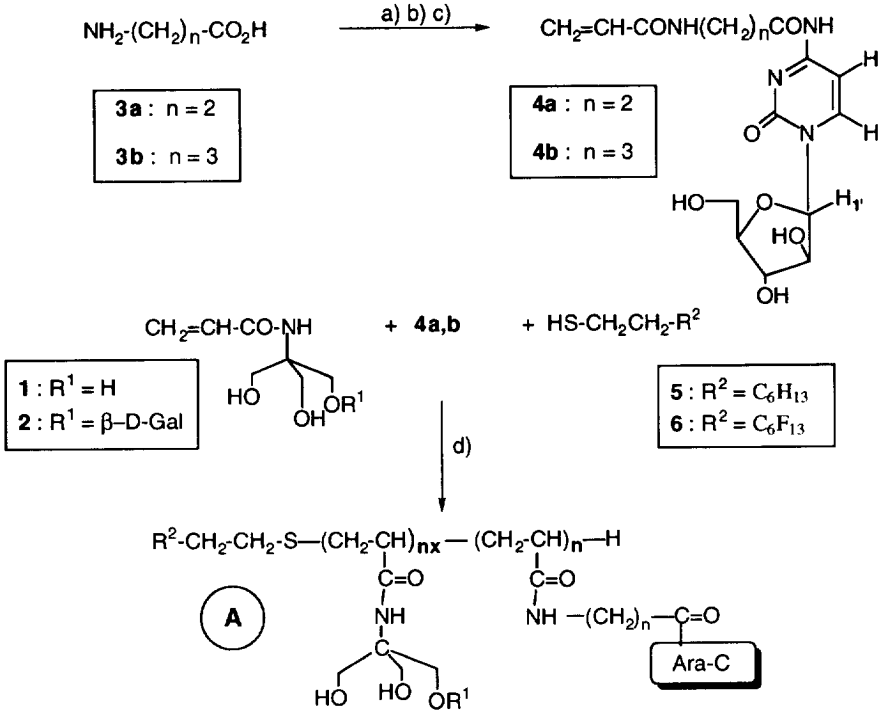
Abstract : The telomerization of tris(hydroxymethyl)acrylamidomethane (THAM) and cytosine arabinoside (Ara-C) polymerizable derivatives in the presence of transfer reagent such as alkane or perfluoroalkane thiol is described. The cytotoxic activities of these different compounds are determined on B16, a mouse melanoma cell line. Noteworthy is that all telomers exhibit a biological activity. These preliminary results show that macromolecular carriers such as cotelomers penetrate into the cell and release the drug after an enzymatic hydrolysis. Copyright © 1996 Elsevier Science Ltd

Linking bioactive molecules to macromolecular carriers is routinely used to increase the persistence of drugs in the body and specially in target tissues[1-4]. These large molecules diffuse slowly and are absorbed at distinct pharmacological interfaces. Consequently, polymer drug conjugates can prolong therapy at sustained dosages. Unfortunately most synthetic molecules are poorly absorbed through the gastrointestinal tract which precludes their oral administration.

Recently, a new class of low molecular weight carriers, named telomers, was synthesized by free radical telomerization of a hydrophilic polymerizable monomer such as acryloylaminoacid or tris(hydroxymethyl) acrylamidomethane (THAM) **1** in the presence of an alkane or perfluoroalkane thiol as a transfer reagent, so-called telogen[5-7]. The multifunctionnal molecules obtained by this method exhibit physicochemical properties, biocompatibility and subcellular distribution making them suitable for drug delivery system[8-9].

In the course of our project dealing with the use of telomers as drug carriers, we synthesized and studied the *in vitro* behavior of cotelomers derived from THAM **1** or monogalactosylated THAM **2** (scheme 1) and acrylamide monomer bearing cytosine arabinoside moieties linked by a peptidic spacer arm such as β-alanine (n=2) **3a** or γ-aminobutyric acid (n = 3) **3 b.** The covalent attachment of the bioactive molecule to the hydrocarbon backbone via a biodegradable bond should favour the drug release following the internalization of the conjugate into the cell. Moreover, glycosylation of THAM hydroxyle functions could insure selective cell targeting via specific recognition of pendant carbohydrates by membrane lectins.

Scheme 1 : Synthesis of telomeric carriers derived from THAM.



a) CH_2=CHCOCl, CH_3OH, KOH 1N, 0°C, 2 h.; yield 90-100% ; b) C_6F_5OH, DCC, CH_2Cl_2, 20°, yield 75-80%; c) Ara-C, TEA, C_6H_5N, 80°C, 12h, N_2 ; yield 78-95% ; d) AIBN, DMF, 80°, 12-24h.

A synthetic route to cotelomers derived from THAM and Arabinose Cytosine (Ara-C) is presented in scheme 1. THAM **1** and monogalactosylated THAM **2** synthesis were previously described[6,10-12].

Concerning preparation of monomers derived from Ara-C, first of all we introduced polymerizable function by coupling acryloyl chloride with compounds **3a** or **3b** in a methanolic solution of potassium hydroxide at 0°C within a pH range between 8 and 9. The attachment of the *N*-acrylamido derivatives on the Ara-C hydrochloride is realized in pyridine by using their pentafluorophenyl esters. The Ara-C polymerizable monomers are isolated as a white powder **4a** : yield 95 %, F = 182°C (dec) ; **4b** : yield 78 %, F = 188°C.

Telomerization experiments are performed in dimethylformamide at 80°C under a nitrogen atmosphere using α,α'-(azobis)-isobutyronitrile (AIBN) as initiator at a concentration roughly ten times lower than the telogen[13].The proportions of monomers **4a** or **4b** and of octanethiol telogen **5** or 1H, 1H, 2H, 2H perfluoro octanethiol **6** used are reported in the table 1. These proportions take into account the previous results obtained with THAM telomerization[6,7] . Each experiment is continued until monomers disappeared (12 to 24 h). Telomers are purified by gel chromatography through a Sephadex G 25 column and then lyophilised. The structures of these cotelomers, i.e. the relative proportions of each moiety THAM (nx) or Ara-C (n) in the cotelomer and the DPn of macromolecules, are evaluated by elemental analysis and in ^{1}H-NMR 250 or 400 Mhz (solvent : DMSO d6) by comparing the typical signals of each monomer[14]. The main parameters of the reactions and compounds prepared during this work are reported in table 1.

Table 1. Physico-chemical data and biological activity for cotelomers of general structure **A**.

Line	Name [a]	Reagents [b]		Cotelomers [c]					IC50 [e]
		5	6	R^2	nx	n	Mw	Yield[d]	
1	Ara-C						243		0.039 ± 0.003
2	Acryl Gaba Ara-C **4b**						383		0.19 ± 0.12
3	H-Monoadduct β Ala Ara-C	1	-	C_6H_{13}	-	1	514	75	0.245 ± 0.042
4	F-Monoadduct Gaba Ara-C	-	1	C_6F_{13}	-	1	762	72	0.37 ± 0.045
5	H-THAM β Ala Ara-C (1/1)	1/8	-	C_6H_{13}	5.5	3.2	2286	57	0.021 ± 0.005
6	F-THAM β Ala Ara-C (1/1)	-	1/8	C_6F_{13}	7.5	5.5	7700	47	0.06 ± 0.01
7	H-THAM β Ala Ara-C (4/1)	1/8		C_6H_{13}	8	1	1928	83	0.044 ± 0.008
8	F-THAM Gaba Ara-C (4/1)	-	1/8	C_6F_{13}	8.5	1.1	2284	50	0.078 ± 0.004
9	H-THAM Monogal βAla Ara-C (1/1)	1/8	-	C_6H_{13}	5.5	3.6	3313	80	0.28 ± 0.02
10	F-THAM Monogal Gaba Ara-C (4/1)	-	1/8	C_6F_{13}	8.5	1	3606	60	0.78 ± 0.14

a) Molar ratio of each monomer used for the cotelomerization are indicated in brackets.
b) Molar ratio of telogen agent based on the monomer concentration.
*c) nx and n are respectively the average number of monomer derived from THAM **1** or **2** and Ara-C **4a** or **4b** in the cotelomer, (n(x+1)= Dpn).*
d) Yields of telomerization reactions.
e) IC-50= concentrations of telomer (expressed in μM) giving a 50% efficiency compared to untreated melanoma cells. Each value is the average of three experiments.

Cytotoxicity assays are performed using the colony forming method on cell line B16[15], a mouse melanoma cell line. The anti-proliferative activity is expressed as IC 50 (inhibitory concentration 50%), the drug concentration giving a 50% cloning efficiency compared to untreated cells. Results are summarized in Table 1.

Compounds described in this table deserve several comments :

1/ The modifications given to the Ara-C molecule decrease its cytotoxicity (lines 2 to 4). These results lead us to think that the different chain attached to the drug may partially inhibit the transmembranar crossing of the substrate. Since synthesized compounds are stable within a pH range from 4 to 10 in aqueous solution, an enzymatic hydrolysis of the amide bond should take place after endocytose.

2/ The monoadduct or oligomers of the Ara-C perfluoroalkyl derivatives show in all cases a cytoxicity slightly weaker than the one observed with theirs hydrocarbon homologous. These results can be explained by the amphiphilic character of cell bilayer and telomers. An hydrocarbon tail can exhibit an affinity for the phospholipidic bilayer and therefore should favour the endocytose process ; this is not the case of the perfluoroalkyl chains.

3/ The compounds obtained during the cotelomerization reactions in the presence of THAM exhibit a cytotoxic activity clearly higher than the monoadducts and, at least, equivalent to the one observed with the authentic Ara-C (lines 5 to 8). The difference of efficiency between the cotelomers and monoadducts could be attribuated to a cluster effect.

4/ The cotelomer activity seems to depend on the THAM glycosylation which decrease their cytotoxicity. Therefore, one can suggest that the galactosylation inhibits partially either the macromolecular endocytose or the intracellular hydrolysis which induces the drug release. We are synthesizing new labeled telomers derived from THAM and Ara-C monomers to understand and find a good explanation to this phenomenon.

Further investigations are underway to explore and rationalize the potentialities of such carriers *in vitro* and *in vivo*.. A particular attention is given to the therapeutic index of such telomers, i.e to assess their cytotoxicity and toxicity *in vivo* with regard to that of Ara-C.

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14/ *For example, x is determined by comparing peak areas assigned to the H1' proton of the ARA-C ribose (δ = 6.05 ppm ; integral = n H) with the THAM hydroxyle protons (δ = 5, ppm, integral = 3 nx H).For the telomers bearing a hydrocarbon tail, n is evaluated by comparing the signal area due to the three protons of the terminal methyle (δ = 0.85 ppm ; Integral = 3H) to the area of the ribose proton H1'. In the case of fluorocarbon compounds, the elemental analysis allow us to calculate the ratio %N/%F. Keeping in mind the x previously evaluated, n is determined following the equation :*

$$\frac{\%N}{\%F} = \frac{n.14(x+4)}{13.19}$$

15/ *B16 cell line is a mouse melanoma cell line derived from spontaneous skin tumor in C57BL/6 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% 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 hr 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 CO_2 incubator. After this time, all dishes were rinsed with phosphate buffer saline 0,05M pH 7,4, cells were fixed with methanol and stained with 0,2% crystal violet solution and colonies (>50 cells) were counted.*

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