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Original article

In vitro and in vivo evaluations of THAM derived telomers bearing RGD and Ara-C for tumour neovasculature targeting

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Abstract

As an approach to the development of specific drug delivery systems, a new class of low macromolecular carriers called 'telomers' endowed with an antitumour agent, such as arabinofuranosylcytosine (Ara-C), RGDSK peptidic sequences, as tumour targeting moieties, and tyrosine groups labelled with ^{125}I atoms allowing the in vivo scintigraphic follow up, were synthesized. Their tumour targeting ability was assessed in vivo in mice bearing a murine B16 melanoma. The biological results showed that the presence of RGDSK sequences onto the macromolecules leads to the selective targeting and the accumulation of telomers within the vascularized zone of the tumour. Moreover, such compounds exhibited in vitro a better IC_{50} (0.015 μM) than pure Ara-C and in vivo an oncostatic index higher than 160%.

Keywords: RGD; Tris; Telomers; Prodrugs; Arabinofuranosylcytosine; Angiogenesis

1. Introduction

Currently, a large variety of chemotherapeutic drugs are widely used to treat malignant diseases. Unfortunately, the remarkable antitumour activity exhibited by many cytotoxic drugs is accompanied by undesirable side-effects causing severe damage to healthy tissues and therefore, limiting their efficacy [1,2]. In this context, targeting of therapeutics to specific tumoural sites could increase their biological effect and reduce their whole toxicity.

Delivery of cytotoxics to tumour cells is a problematic challenge because of their genomic instability (i.e. gene amplification, chromosomal translocations, chromosome loss ...), heterogeneity and rapid mutation leading to the development of drug resistance [3]. Acquired drug resistance has been identified as the major cause of inefficacy of cytotoxic tumour therapy [4]. In contrast, the endothelial cells lining the tumour vessels consist of a homogeneous and genetically stable population less likely to develop resistance to a particular agent [5]. Targeting the tumour vasculature rather than the tumour cell population itself could also circumvent one of the major mechanism of drug resistance involving insufficient drug uptake by the tumour mass.

In fact, a tumour cannot grow beyond the size of 1–2 mm in diameter without developing a blood supply providing oxygen and nutrients. This phenomenon, known as angiogenesis, was hypothesized 30 years ago, by Judah Folkman, to be an absolute requirement for the growth and metastasis of solid tumours [6,7].

Abbreviations: AIBN, α,α′-azobisisobutyronitrile; Ara-C, arabinofuranosylcytosine; β-Ala, β-Alanine; BOP, benzotriazol-1-yl-oxy-tris(dimethylamino)-phosphonium hexafluorophosphate; DCC, 1,3-dicyclohexylcarbodiimide; DCU, 1,3-dicyclohexylurea; DIEA, diisopropylethylamine; DPn, number average degree of polymerization; ECM, extracellular matrix; Fmoc, 9-fluorenylmethoxycarbonyl; Gaba, γ-amino butyric acid; i.v., intravenous; p.o., per os; SAR, structure-activity relationship; TBTU, *O*-(1*H*-benzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium tetrafluoroborate; TEA, triethylamine; THAM, tris(hydroxymethyl)acrylamidomethane; Tris, tris(hydroxymethyl)aminomethane; Mtr, 2,3,6-trimethyl-4-methoxy-benzenesulfonyl.

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If in one hand the easy accessibility of the tumour microenvironment may provide therapeutic opportunities, on the other hand, tumour blood vessels exhibit several abnormalities in comparison with normal physiological vessels [8] which could also constitute potential targets for specific antiangiogenic therapy. Indeed, the vasculature undergoing angiogenesis differs from normal quiescent vasculature by an overexpression of ECM-binding integrin receptors such as αVβ3 and αVβ5. Other specific markers including adhesion molecules like E-selectin or VEGF receptors are upregulated on activated endothelial cells, making them suitable targets for chemotherapeutics administration [9,10]. Many of these phenotypic differences can be exploited not only as potential therapeutic targets but also to establish an 'angiogenic profile' of patients with cancer or exhibiting other chronic angiogenic diseases. In this regard, it is today possible to discriminate newly formed vessels from those that are more mature using specific antibodies [11] or by medical imaging techniques based on nuclear medicine. Several studies indicate that appropriate integrin activation on endothelial cells is required for maturation of angiogenic vessels. Intense researches have been focused on αVβ3 integrin and showed that its blockade during angiogenesis process results in apoptosis of activated endothelial cells and a marked decreased of angiogenesis [12]. An example is the use of specific antagonists of αVβ3 receptors such as monoclonal antibody LM 609 or small cyclic RGD-containing peptides: these compounds induce rapid apoptosis only in newly sprouting vessels, without affecting mature preexisting vessels [13,9].

This affinity of RGD peptides for αVβ3 receptors expressed on tumour blood vessels and on some tumoural cells has found major attention. Recently, this property has been exploited to image tumours in vitro and in vivo using radiolabelled RGD-containing peptides [14,15].

All of these interesting data suggested us that an increased efficiency could be achieved by grafting simultaneously a cytotoxic agent such as Ara-C and a RGD-containing oligopeptide onto a carrier. A pioneering work in this field was previously reported by Ruoslahti and co-workers [16] who have coupled doxorubicin with a cyclic RGD peptide.

In an attempt to provide a selective tumour cells targeting, we chose to introduce RGD moieties on the low macromolecular drug delivery systems we have been developing from many years in our team, namely, 'telomers' [17]. By this way, we can expect to obtain telomeric prodrugs of Ara-C exhibiting an increased affinity for the highly vascularized zone surrounding tumours. Furthermore, tumour-associated vasculature is known to be hyperpermeable to macromolecules such as plasma proteins and other high molecular blood components [8,18]. Different authors showed that tumour microvasculature is more leaky to macromolecular tracers than that of comparable normal tissues [19]. In this context, aside from reducing the toxicity of Ara-C on normal cells by a selective targeting of angiogenic sites, we could assume that telomers bearing both Ara-C and RGD units could concentrate in tumour vessels easier than free Ara-C.

Work reported herein deals with the synthesis and preliminary biological assessments of telomeric prodrugs of Ara-C allowing the selective targeting of proliferative angiogenic vascular cells supporting tumours growth.

2. Chemistry

The strong implication of the integrin receptor αVβ3 in the development of solid tumours and metastatic colonies has been widely demonstrated [20,21]. Among the numerous peptidic ligands of this receptor, the so-called 'universal cell recognition sequence' RGD (Arg-Gly-Asp) has been one of the most studied [22,23]. This sequence, known to be a recognition element in many integrin-dependent cell adhesion processes, is found in many extracellular matrix proteins like vitronectin, laminin, fibrinogen and fibronectin [24]. Although this RGD motif occurs frequently in various proteins, only a small number of them exhibit an integrin-binding activity. Indeed, it should be noted that various RGD-containing peptides, exhibiting a RGD sequence inaccessible to integrins, present a limited integrin-binding activity [25].

Many SAR investigations, performed with peptides selected from random phage display screens [25,26] or with synthetic linear or cyclic peptides [27], have shown that the amino acids flanking the recognition motif influence the affinity of the whole RGD-containing ligand toward the αVβ3 receptor. In particular, hydrophobic amino acids following the aspartic acid enhance this affinity. However, small synthetic RGD-peptides with serine in position 4 showed a very high inhibitory activity on the vitronectin-αVβ3 interaction. Many authors suggested that a hydrogen bond between the hydroxyl group of serine and an acceptor group of the receptor might be formed in addition to the hydrophobic interactions that also take place in this region [28,29]. With regard to the amino acid in position 5, neither hydrophobic nor hydrophilic parameters seem to influence the activity. Considering all these data, and to provide a good integrins accessibility for the RGD moieties, we decided to synthesize a peptidic chain containing a RGDSK sequence. This pentapeptide was grafted onto the telomer backbone through a γ-aminobutanoic acid spacer arm.

The telomeric carriers, short macromolecules bearing a functionnalized end-group, are usually obtained by

free radical copolymerization of acryloyl monomers derived both from tris(hydroxymethyl)acrylamidomethane (THAM) and acryloyl peptide in the presence of an alkane or a perfluoroalkanethiol as a transfer reagent. In previous publications, we reported that the physico-chemical parameters of these telomers (molecular weight, Hydrophilic Lipophilic Balance, electric charge) can be adjusted through both the starting material and the experimental conditions [30,31]. As regards their biological properties, we have shown that these multifunctional carriers are able to cross physiological membranes [32] to diffuse in all tissues. A whole body autoradiography performed in rat and mouse allowed us to specify the quite ubiquitous biodistribution of THAM derived telomers in all biological compartments except brain [33]. Moreover, after intravenous (i.v.) or per os (p.o.) administration to rat, these compounds were slowly released within 100 h and no toxicity was observed. Finally, the anchorage of various antitumour agents such as cytosine arabinoside (Ara-C) or 5-fluorouracil (5-Fu) to the polymeric backbone gave macromolecular prodrugs exhibiting, in vitro and in vivo, an increased bioavailability and a better therapeutic index than the parent drugs [34–36].

The RGDSK sequence **4** was prepared following conventional methods of liquid phase synthesis applying fmoc-strategy (Fig. 1) [37] In order to perform the final peptide deprotection by using acid hydrolysis, suitable labile groups were chosen to protect the different amino acids: *t*-butyl group for serine and aspartic acid, Mtr group in the case of arginine. Compounds **1** and **4** were obtained using respectively BOP and TBTU coupling reagents in methylene chloride. Pentafluorophenol and DCC were both used to carry out the synthesis of synthons **5** and **6**. Following this pathway, all compounds were fully characterized by NMR spectroscopy. The RGDSK protected sequence was prepared with an overall yield of 43% and gave satisfactory mass spectrometric analysis.

In order to follow-up the macromolecules in vivo, a tyrosine polymerizable monomer **7**, easily radiolabelled with ^{125}I atoms, was used during the preparation of THAM derived cotelomers **11**, **12** and **13**. The synthesis of this monomer **7** started by activating the *N*-γ-acrylamidobutyric acid as its hydroxysuccinimidyl ester **9**. Consecutive coupling of this active ester to *O*-(*tert*-butyl) *tert*-butyl L tyrosinate was performed in methylene chloride at room temperature (r.t.) and provided monomer **7** in satisfactory yield (87%).

Peracetylated THAM monomer **8** was easily synthesized from THAM using the conventional reactant acetic anhydride in pyridine at r.t. for 12 h. Compound **8** was obtained after recrystallization in an ethyl acetate–hexane mixture as a white powder (87% yield). The preparation of the Ara-C polymerizable monomer **10** was described in a previous paper [35].

These four polymerizable monomers **7**, **8**, **9**, **10** gave satisfactory elemental analysis and were fully characterized by NMR spectroscopy.

Telomerization of such monomers, providing amphiphilic telomers **11a** (then **11**), **12a** (then **12**) and **13a** (then **13**), was performed as previously described [33,38] in the presence of alkanethiol as transfer reagent (or telogen), using AIBN as radical initiator (see Fig. 2). The AIBN concentration in the reaction mixture was roughly 10 times lower than the telogen one [39]. The number average degree of polymerization (DPn) is equal to the amount of repeating units $(x+y+z+w)$ or $(x+y+v+w)$. With a given transfer reagent, it may vary from one (monoadduct) to several tens, depending on the (telogen)/(monomers) ratio (R_0) adjusted through both starting material and experimental conditions [30]. The proportions of monomers **7**, **8**, **9** and/or **10** and octanethiol used are reported in Table I. These proportions were chosen taking into account previous results obtained with THAM telomerization [38,31]. Each experiment was pursued until the complete disappearance of the monomers. Telomers were purified by chromatography through a Sephadex LH20 column (for compounds **11a**, **12a** and **13a**) or a Sephadex G50 column and then lyophilized (compounds **11**, **12**, **13**). The hydrolysis of *tert*-butyl protective groups of tyrosine pendants on compound **11a**, by using a trifluoroacetic acid–methylene chloride mixture (3/7 v/v), provided telomer **11** in satisfactory yields. With regard to the grafting of the peptidic chain to telomers **12a** and **13a**, the benzyloxycarbonyl protection of the side-chain amino group of lysine in RGDSK sequence **4** was first removed by catalytic hydrogenolysis on Pd/C. Consecutive coupling of this peptide **4a** to the active ester groups of telomers **12a** and **13a** was carried out in methylene chloride at r.t. for 24 h under a nitrogen atmosphere. The structure of these cotelomers **11a** (or **11**), **12a** (or **12**) and **13a** (or **13**), i.e. the relative proportions of each tyrosine (x), peracetylated THAM (y), active ester or RGD (z or v) and/or Ara-C (w) moiety in the cotelomer and the DPn of the macromolecule, were determined in ^{1}H-NMR by comparing the area of typical signals of each monomer.

For example, in the case of telomer **11**, x, y and w values were determined by comparing peaks area assigned to the terminal methyl signal in the hydrocarbon tail (δ 0.9 ppm, integral 3H), respectively to aromatic tyrosine protons (δ 6.89 ppm, integral $2x$H), to methylene protons of peracetylated THAM (δ 4.32 ppm, integral $6y$H) and to H_6 proton of Ara-C moieties (δ 8.14 ppm, integral $1w$H). The respective value of z for telomers **12a** and **13a**, was determined as above, by comparing peaks area assigned to the terminal methyl signal in the hydrocarbon tail (δ 0.9 ppm, integral 3H) to succinimidyl protons of active ester groups (δ 2.86 ppm, integral $4z$H).

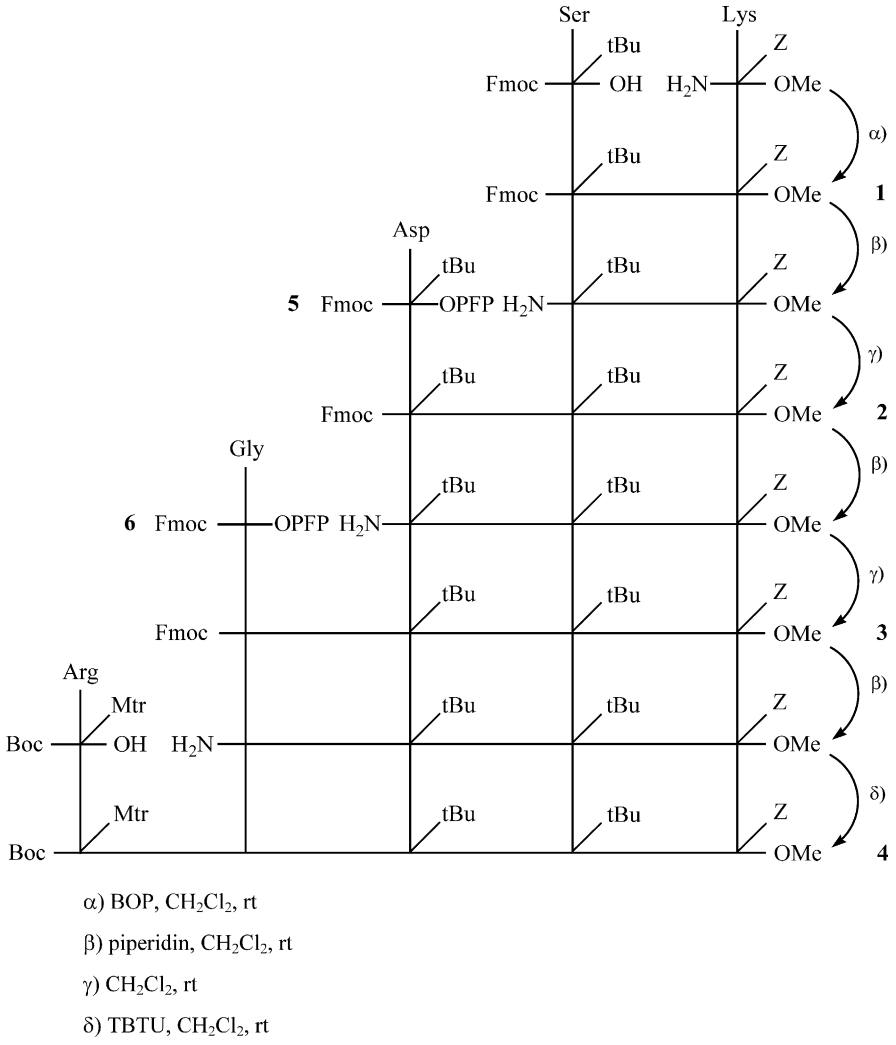

Fig. 1. Synthesis of the RGDSK sequence [37].

The proportion of RGD sequences grafted onto telomers **12** and **13** was specified, before the deprotection of Mtr groups, by comparing in ^{1}H-NMR peaks area assigned to the aromatic Mtr proton (δ 6.56 ppm, 1*v*H) to the terminal methyl signal in the hydrocarbon tail. It is noteworthy that for each telomer, the NMR structural evaluation showed that the proportion of RGD moieties was similar to the succinimidyl groups one in the precursor telomers **12a** and **13a**. Finally, the hydrolysis of both *tert*-butyl and Mtr protective groups, using a trifluoroacetic acid–methylene chloride–methyl phenyl sulfide mixture (3/5/2 v/v/v) at r.t. for 24 h, was checked by ^{1}H-NMR spectrum after a purification step and gave compounds **12** and **13** in good conditions.

3. Pharmacology

A tyrosine residue was introduced in the chemical structure of telomers in order to perform a labelling of the molecules with ^{125}I. Using these labelled compounds, their biodisposition was studied in mice bearing syngenic B16 melanoma tumours. The pharmacological activity of the molecules **11** and **13** bearing Ara-C was studied on B16 melanoma in vitro, using the measure of the coloning efficiency (colony forming) and in vivo, by determining the oncostatic index after treatment by Ara-C equivalent doses of **11** and **13** on mice bearing grafted B16 melanoma.

4. Results and discussion

Tumour growth and metastasis have been proved to be dependent on the development of new capillaries from preexisting blood vessels, namely ‘angiogenesis’. Many authors reported that angiogenesis depends on specific endothelial cell adhesive events mediated by integrin αVβ3 [40,41] which presents a high affinity for RGD peptides.

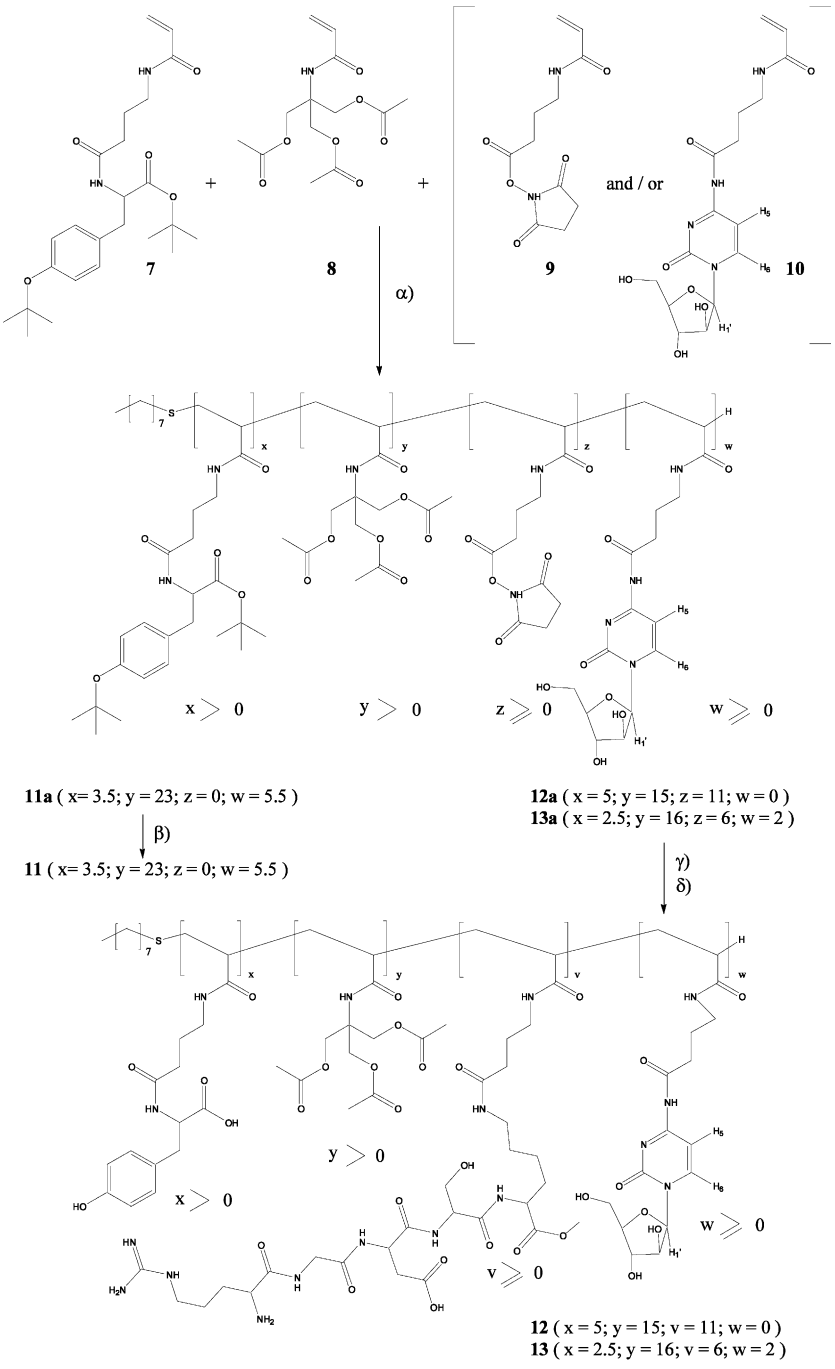

Fig. 2. Synthesis of telomeric carriers derived from THAM.

In this context, we expected that the introduction of RGD pendants on our macromolecular carriers called 'telomers' could be a convenient approach for the selective delivery of chemotherapeutics to tumoural zones.

A series of three amphiphilic telomers radiolabelled by ^{125}I was then synthesized. Different structural parameters likely to enhance the potency of the prodrug towards tumoural tissues have been considered and their influence evaluated. Among them, for roughly similar

Table I
Experimental data of the cotelomers synthesis

Compounds	Initial Conditions [monomer]/[telogen]				Solvent	Telomer structures							
	7	**8**	**9**	**10**		*x*	*y*	*z*	*w*	DPn	M_w	Yield (%)	Compound/final M_w
11a	5	15	0	10	DMF	3.5	23	0	5.5	32	10 700	73	**11**/10 300
12a	8	14	6	0	THF	5	15	11	0	31	9600	75	**12**/14 100
13a	5	20	10	10	DMF	2.5	16	6	2	26.5	8300	54	**13**/10 800

x, *y*, *z*, and *w* are the average numbers derived from tyrosine (*x*), peracetylated THAM (*y*), active ester or RGD (*z*) and Ara-C (*w*) moiety in the cotelomers.DPn = number average degree of polymerization equal to $x+y+z+w$.

telomeric sizes (i.e. their DPn), we studied the effect of the number of RGD pendants and the multiplicity of Ara-C moieties on the macromolecular backbone.

All compounds were well soluble in water and so, were likely to be administered to mice by i.v. injection. Their biodistribution was specified by measuring the radioactivity levels in several tissues as a function of time following administration.

Photograph 1 and results reported in Table II clearly show the significant affinity of telomers **12** and **13**, bearing RGDSK sequences, for the vascularized zone of the tumour while telomer **11** does not show any affinity. Indeed, for these compounds, the ratio stroma/blood is, whatever the time, always higher than 1. Comparatively, telomer **11**, without any RGDSK moieties, does not concentrate in these compartments and so exhibits no affinity for neither angiogenic nor tumoural sites (Table II). This lack of affinity of telomer **11** for specific compartments of the body is in good agreement with the homogeneous biodistribution of radiolabelled telomers derived from THAM previously observed [33].

Moreover, if we consider the relative proportion of telomers **12** and **13** in each tumoural and stroma zone, we can note an increase of the ratio tumour/stroma versus time, underlining the diffusion of these prodrugs toward the tumour mass. However, 6 h after administration, tumoural tissue exhibits slightly lower concentration values for **12** than for **13**, while blood concentration decrease is higher for **13**, probably linked to a faster urinary elimination, as confirmed by the significantly higher kidney radioactivity for **13** at this time. Such an elimination rate could be due to the presence of Ara-C moieties on the telomer backbone which increase its hydrophilic character.

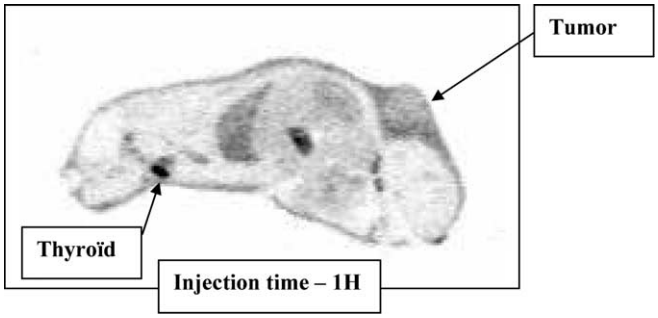

Plate 1. Mouse section 1 h after injection of telomer **13** (bearing RGDSK sequences).

These biological observations demonstrate the key role of RGD moieties for the specific targeting of highly vascularized zones surrounding the tumour. However, by comparing the behaviour of compounds **12** and **13** which were endowed with different RGD sequences number, no cluster effect could be noted. The affinity of these telomers for the vascularized zone cannot be really correlated to the number of RGD groups they own.

The in vitro and in vivo antitumour potencies of the two telomers **11** (without RGDSK sequences) and **13** bearing both Ara-C were investigated on B16 melanoma by measuring the coloning efficiency and the oncostatic index. Fig. 3 shows the dose-effect curves for compounds **11** and **13**. From these results, we determined an IC50 of 0.015 μM for **11** and 0.26 μM for **13**, (comparatively, telomer **12**; without Ara-C units exhibits no cytotoxic activity). Oncostatic indexes were 162% for **11** and 153% for **13**. These results are in accordance with those observed for biodistribution study. Indeed, although **11** was more than 10 fold efficient than **13** for the coloning efficiency test, its oncostatic index was only sligthly superior to this of **13**. This discrepancy between the in vitro and in vivo tests can be explained by a higher concentration of **13** in the tumoural tissue due to the binding of RGD residues to angiogenic zones.

In conclusion, all of these biological evaluations validate the potentialities of THAM derived telomers endowed with specific oligopeptidic sequences such as RGD for targeting chemotherapeutics to the activated endothelium supporting the growth of the tumours. The study of their therapeutic activity is currently underway on mice bearing B16 syngenic melanoma and will be reported in a forthcoming publication.

5. Experimental protocols

The progress of the reactions and the homogeneity of the compounds were monitored by thin-layer chroma-

Table II
Distribution of radioactivity in tissues after i.v. administration of 20 μCi of labelled telomers **11**, **12**, **13**.

	Time after administration								
	15 mn			1 h			6 h		
Compounds	**11**	**12**	**13**	**11**	**12**	**13**	**11**	**12**	**13**
Tumour	3.8±0.6	1.74±0.65	3.34±0.88	0.9±0.2	2.14±0.59	1.57±0.17	ND	1.44±0.27	2.02±0.42
Stroma	4.2±0.7	6.34±0.65	6.62±1.55	1.1±0.2	4.56±0.90	3.84±0.17	ND	1.76±0.24	2.02±0.42
Blood	4.9±1.1	3.29±0.28	5.52±1.09	3.4±0.7	1.99±0.22	1.56±0.24	1.2±0.3	1.09±0.08	0.65±0.11
Skin	ND	3.36±0.56	3.64±0.58	ND	1.48±0.20	1.45±0.32	ND	0.95±0.11	ND
Kidney	6.8±0.4	31.4±5.26	25.72±5.05	4.7±0.5	18.5±3.19	32.80±2.98	2.2±0.3	8.01±1.54	13.80±3.04
Liver	2.9±0.3	2.11±0.35	3.02±0.57	2.1±0.4	0.94±0.17	1.33±0.54	1.5±0.4	0.52±0.12	0.45±0.2
Thyroid	7.5±1.2	8.57±1.15	21.58±2.65	10.7±2.1	54.5±10.2	65.56±9.27	14.4±3.7	75.4±15.2	85.82±14.12
Stroma/blood	0.85	1.92	1.20	0.32	2.29	2.46	ND	1.61	3.10
Tumour/stroma	0.90	0.27	0.50	0.81	0.47	0.40	ND	0.82	1

Results, given in percentage of administered doses per g of tissue, are the mean of five experiments ±SD.

tography (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 chromatography over silica gel (Merck 60), or by gel-permeation on Sephadex G50 or LH20 (Pharmacia LKB). Melting points (m.p.) 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. Elemental analysis were conducted by the 'Service Central de Microanalyse du CNRS' at Lyon (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, N^{γ}-acrylamidobutyric acid and N-(3-(N^4-carbamoyl-1-β-D-arabino-furanosylcytosine)-propyl acrylamide (compound **10**) were synthesized as described by Pucci et al. [31,35].

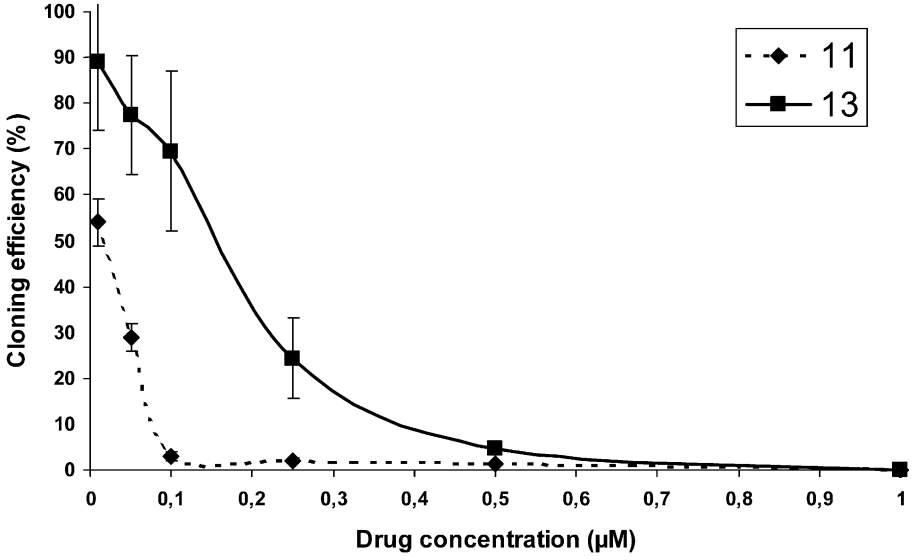

Fig. 3. Coloning efficiency measured on B16 melanoma cells as a function of **11** and **13** concentrations. Each value is the mean of four experiments ± SD.

6. Synthesis of RGDSK sequence [37]

6.1. Fmoc-Ser(tBu)-Lys(Z)-OMe (1)

To a solution of Cl^- ^+H_3N-Lys(*Z*)-OMe (0.99 g, 3 mmol) and DIEA (0.39 g, 3 mmol) in CH_2Cl_2 (10 mL) were successively added Fmoc-Ser(*t*Bu)-OH (1.15 g, 3 mmol) and BOP (1.72 g, 3,9 mmol). The pH was maintained between 8 and 9 and the reaction mixture left at r.t. overnight. The organic phase was then washed with 1 N HCl (2 × 10 mL), saturated aq. NaHCO3 (2 × 10 mL) and water (2 × 10 mL). Drying on Na_2SO_4 and removal the solvent in vacuo afforded a white powder which was used without further purification (1.93 g, 98%): m.p. 60.1 °C (dec.); $[\alpha]_D$ +17.9 (*c* 1, CH_2Cl_2); ^{1}H-NMR ($CDCl_3$): δ 7.75 (d, 2H, arom. Fmoc), 7.60 (d, 2H, arom. Fmoc), 7.43-7.21 (m, 5H, arom. Fmoc, NH Lys), 7.31 (s, 5H, arom. benzyl Lys), 5.78 (d, 1H, NH Ser), 5.08 (s, 2H, CH_2 benzyl Lys), 4.84 (t, 1H, NH Z Lys), 4.60 (m, 1H, H_α Lys), 4.39 (d, 2H, CH_2 Fmoc), 4.24 (m, 2H, H_α Ser, CH Fmoc), 3.81 (dd, 1H, H_β Ser), 3.73 (s, 3H, CH_3 methyl ester Lys), 3.39 (dd, 1H, H_β Ser), 3.17 (m, 2H, $2H_\varepsilon$ Lys), 1.85-1.29 (m, 6H, $2H_\beta$ Lys, $2H_\gamma$ Lys, $2H_\delta$, Lys), 1.22 (s, 9H, 3 CH_3 *tert*-butyl ether Ser); ^{13}C-NMR ($CDCl_3$): δ 172.94 (CO Lys), 170.88 (CO Ser), 157.08 and 156.67 (2 CO urethane Fmoc and Z), 144.52, 144.36, 141.93, 137.23, 129.13, 128.71, 128.35, 127.71, 125.76 and 120.62 (C arom. Fmoc and Z), 74.96, 27.99 (*tert*-butyl ether Ser), 67.80 and 67.24 (CH_2 Fmoc and CH_2 Z), 62.39 (C_β Ser), 54.93 (C_α Ser), 52.99 and 52.74 (C methyl ester Lys and C_α Lys), 47.76 (CH Fmoc), 41.27 (C_ε Lys), 32.74 (C_δ Lys), 30.02 (C_β Lys), 22.92 (C_γ Lys).

6.2. Fmoc-Asp(tBu)-Ser(tBu)-Lys(Z)-OMe (2)

Dipeptide **1** (1.3 g, 1.97 mmol) has been treated with piperidine (10% in CH_2Cl_2) (10 mL) for 1 h at r.t. The organic phase was then washed with 1 N HCl (2 × 10 mL) or until pH 1, saturated aq. NaHCO3 (2 × 10 mL) and water (2 × 10 mL). Drying on Na_2SO_4, the solvent was evaporated under vacuum. To a stirred mixture of the resulting amine in CH_2Cl_2 (10 mL) were added DIEA (pH 8), active ester **6** (1.14 g, 1.97 mmol) and the mixture was stirred overnight at 25 °C. The solution was then concentrated under reduce pressure. The residue was purified by column chromatography on silica gel using ethyl acetate–hexane (50:50 v/v) as eluent to provide the pure product **2** as a white powder (0.97 g, 59.2%); m.p. 68.4 °C (dec.); $[\alpha]_{20}^{D}$ +8.6 (*c* 1, CH_2Cl_2); ^{1}H-NMR ($CDCl_3$): δ 7.78 (d, 2H, arom. Fmoc), 7.61 (d, 2H, arom. Fmoc), 7.46-7.23 (m, 6H, arom. Fmoc, NH Lys, NH Ser), 7.36 (s, 5H, H arom. benzyl Lys), 5.93 (d, 1H, NH Asp), 5.11 (s, 2H, CH_2 Z Lys), 4.91 (t, 1H, NH Z Lys), 4.57 (m, 2H, H_α Asp, C_α Lys), 4.48-4.42 (m, 3H, H_α Ser, CH_2 Fmoc), 4.26 (t, 1H, CH Fmoc), 3.85 (dd, 1H, H_β Ser), 3.74 (s, 3H, CH_3 methyl ester Lys), 3.41 (dd, 1H, H_β Ser), 3.17 (m, 2H, $2H_\varepsilon$ Lys), 2.88 (dd, 1H, H_β Asp), 2.72 (dd, 1H, H_β Asp), 1.48 (s, 9H, 3 CH_3 *tert*-butyl ester Asp), 1.84-1.28 (m, 6H, $2H_\beta$, $2H_\gamma$, $2H_\delta$ Lys), 1.22 (s, 9H, 3 CH_3 *tert*-butyl ether Ser); ^{13}C-NMR ($CDCl_3$): δ 172.96, 171.74, 171.14 and 170.52 (CO Lys, CO Ser, 2 CO Asp), 157.06 and 156.65 (2 CO urethane Fmoc and Z), 144.32, 141.94, 137.28, 129.14, 128.71, 128.41, 127.76, 125.72 and 120.65 (C arom. Fmoc and Z), 82.59-28.67 (*tert*-butyl ester Asp), 74.80, 27.97 (*tert*-butyl ether Ser), 68.04 and 67.88 (CH_2 Fmoc and CH_2 benzyl Z), 61.61 (C_β Ser), 53.99 (C_α Ser), 52.95 and 52.70 (C methyl ester Lys and C_α Lys), 52.04 (C_α Asp), 47.74 (CH Fmoc), 41.32 (C_ε Lys), 38.25 (C_β Asp), 32.55 (C_δ Lys), 29.93 (C_β Lys), 22.99 (C_γ Lys); MS (FAB): *m*/*z* 831 $[M+H]^+$, 853 $[M+Na]^+$.

6.3. Fmoc-Gly-Asp(tBu)-Ser(tBu)-Lys(Z)-OMe (3)

To a solution of the tripeptide resulting from Fmoc deprotection of **2** (0.5 g, 0.6 mmol) in 10 mL of piperidine–methylene chloride (1:9, v/v), was added active ester **6** (0.28 g, 0.6 mmol) and DIEA (pH 8–9) in CH_2Cl_2 (10 mL). The stirring was pursued for 16 h at r.t. The solution was then evaporated under reduced pressure and the crude product was purified by chromatography (EtOAc–hexane, 60:40, v/v) to give a white powder **3** (0.43 g, 80%); m.p. 53.3 °C (dec.); $[\alpha]_{20}^{D}$ +4.6 (*c* 1, CH_2Cl_2); ^{1}H-NMR ($CDCl_3$): δ 7.76 (d, 2H, arom. Fmoc), 7.60 (d, 2H, arom. Fmoc), 7.45-7.22 (m, 7H, arom. Fmoc, NH Lys, NH Ser, NH Asp,), 7.34 (s, 5H, arom. benzyl Lys), 5.83 (d, 1H, NH Gly), 5.14 (t, 1H, NH Z Lys), 5.07 (s, 2H, CH_2 benzyl Lys), 4.82 (m, 1H, H_α Asp), 4.57 (m, 1H, H_α Lys), 4.45-4.35 (m, 3H, H_α Ser, CH_2 Fmoc), 4.20 (t, 1H, CH Fmoc), 3.89 (m, 2H, $2H_\alpha$ Gly), 3.75 (dd, 1H, H_β Ser), 3.68 (s, 3H, CH_3 methyl ester Lys), 3.39 (dd, 1H, H_β Ser), 3.15 (m, 2H, $2H_\varepsilon$ Lys), 2.88 (dd, 1H, H_β Asp), 2.68 (dd, 1H, H_β Asp), 1.41 (s, 9H, 3 CH_3 *tert*-butyl ester Asp), 1.91-1.29 (m, 4H, $2H_\beta$, $2H_\gamma$ Lys), 1.22 (s, 9H, 3 CH_3 *tert*-butyl ester Ser); ^{13}C-NMR ($CDCl_3$): δ 173.00, 171.90, 170.75, 170.49, 169.76 (CO Lys, CO Ser, 2 CO Asp, CO Gly), 157.31 and 157.13 (2 CO urethane Fmoc and Z), 144.45, 141.98, 137.27, 129.19, 128.78, 128.42, 127.78, 125.77, 120.68 (C arom. Fmoc and Z), 82.78, 28.67 (*tert*-butyl ester Asp), 74.78, 28.00 (*tert*-butyl ether Ser), 68.05 and 67.30 (CH_2 Fmoc and CH_2 benzyl Z), 61.60 (C_β Ser), 54.19 (C_α Ser), 53.01 and 52.72 (C methyl ester Lys and C_α Lys), 50.28 (C_α Asp), 47.76 (CH Fmoc), 45.26 (C_α Gly), 41.38 (C_ε Lys), 37.68 (C_β Asp), 32.58 (C_δ Lys), 29.99 (C_β Lys), 23.03 (C_γ Lys); MS (FAB): *m*/*z* 888 $[M+H]^+$, 910 $[M+Na]^+$.

6.4. Boc-Arg(Mtr)-Gly-Asp(tBu)-Ser(tBu)-Lys(Z)-OMe (4)

The reaction was carried out using tetrapeptide **3** (0.4 g, 0.45 mmol), which was deprotected in 10 mL of piperidine–methylene chloride (1:9, v/v), Boc-Arg(Mtr)-OH (0.22 g, 0.45 mmol), TBTU (0.19 g, 0.58 mmol) and DIEA until pH 8–9 in CH_2Cl_2 (10 mL). Solvent was evaporated under vacuum and the crude product purified by column chromatography on silica gel using ethyl acetate–hexane (90:10, v/v) as eluent, to provide the pure product **4** as a white powder (0.42 g, 92.5 %): m.p. 80.2 °C (dec.); $[\alpha]_{20}^{D}$ −2.9 (*c* 1, CH_2Cl_2); ^{1}H-NMR ($CDCl_3$): δ 7.64 (d, 1H, NH Asp), 7.53 (d, 1H, NH Gly), 7.49-7.29 (m, 7H, arom. Z Lys, NH Lys, NH Ser), 6.54 (s, 1H, H arom. Mtr), 6.34-6.15 (m, 3H, 3 NH guanidine Arg), 5.65 (d, 1H, NH Boc Arg), 5.24 (t, 1H, NH Z Lys), 5.10 (s, 2H, CH_2 Z Lys), 4.77 (m, 1H, H_α Asp), 4.46 (m, 2H, H_α Lys, H_α Ser), 4.22 (m, 1H, H_α Arg), 3.89-3.70 (m, 3H, $2H_\alpha$ Gly, H_β Ser), 3.84 (s, 3H, CH_3 ether Mtr Arg), 3.71 (s, 3H, CH_3 methyl ester Lys), 3.50 (dd, 1H, H_β Ser), 3.21 (m, 2H, $2H_\delta$ Arg), 3.17 (m, 2H, $2H_\varepsilon$ Lys), 2.90-2.64 (m, 8H, 2 CH_3 Mtr Arg, $2H_\beta$ Asp), 2.15 (s, 3H, CH_3 Mtr Arg), 1.84-1.19 (m, 37H, 9 CH_3 *tert*-butyl ester Asp, *tert*-butyl ether Ser, *tert*-butyl Boc Arg, $2H_\beta$, $2H_\gamma$, $2H_\delta$ Lys, $2H_\beta$, $2H_\gamma$ Arg); ^{13}C-NMR ($CDCl_3$): δ 174.32, 174.13, 172.85, 171.48, 171.35, 170.92, 170.18 (CO Lys, CO Ser, 2 CO Asp, CO Gly, CO Arg, C guanidin Arg), 158.96, 157.20 and 156.55 (2 CO urethane Boc and Z, C–OCH_3 arom. Mtr Arg), 139.07, 137.25, 137.06, 134.19, 129.07, 128.87, 128.61, 125.30, 112.28 (C arom. Mtr and Z), 82.37 (C *tert*-butyl ester Asp), 80.53 (C *tert*-butyl Boc Arg), 74.63 (C *tert*-butyl ether Ser), 67.09 (CH_2 benzyl Lys), 60.96 (C_β Ser), 55.99 (C methyl ether Mtr Arg), 54.57 and 54.23 (C_α Ser, C_α Arg), 52.87 and 52.74 (C methyl ester Lys and C_α Lys), 50.37 (C_α Asp), 43.92 (C_α Gly), 41.25 and 40.75 (C_ε Lys, C_δ Arg), 37.59 (C_β Asp), 32.27 (C_δ Lys), 30.33 and 29.83 (C_β Lys and C_β Arg), 28.94, 28.56, 27.86 (9 C methyl *tert*-butyl Asp, Ser and Arg), 25.93 (C_γ Arg), 24.74 (C_γ Lys), 23.06, 18.95, 12.52 (3 C methyl Mtr Arg); MS (FAB): *m/z* 1135 $[M+H]^+$, 1157 $[M+Na]^+$.

6.5. Fmoc-Asp(tBu)-OPFP (5)

Pentafluorophenol (0.68 g, 3.66 mmol) was added to a solution of Fmoc-Asp(*t*Bu)-OH (1.36 g, 3.06 mmol) and DCC (0.76 g, 3.66 mmol) in methylene chloride. The mixture was stirred for 15 h at r.t. and the precipitated DCU was filtered. The filtrate was evaporated under vacuum and the residue crystallized with ethyl acetate/heptane to give **5** (1.52 g, 86.2%); m.p. 94.6–95.8 °C; $[\alpha]_{20}^{D}$ −2.3 (*c* 1, CH_2Cl_2); ^{1}H-NMR ($CDCl_3$): δ 7.77 (d, 2H, arom. Fmoc), 7.61 (d, 2H, arom. Fmoc), 7.43-7.28 (m, 4H, arom. Fmoc), 5.98 (d, 1H, NH), 4.98 (m, 1H, H_α Asp), 4.49-4.23 (m, 3H, CH_2 Fmoc, CH Fmoc), 3.15 (dd, 1H, H_β Asp), 2.90 (dd, 1H, H_β Asp), 1.48 (s, 9H, 3 CH_3 *tert*-butyl ester Asp); ^{13}C-NMR ($CDCl_3$): δ 170.35 and 168.09 (2 CO ester), 156.55 (CO urethane Fmoc), 144.43, 144.26, 142.01, 128.47, 127.78, 125.79, 120.71 (C arom. Fmoc), 143.82-136.34 (CF), 83.39-28.68 (*tert*-butyl ester Asp), 68.21 (CH_2 Fmoc), 50.97 (C_α Asp), 47.76 (CH Fmoc), 38.31 (C_β Asp); ^{19}F-NMR ($CDCl_3$): δ −152.22 (2F), −157.36 (1F), −162.10 (2F).

6.6. Fmoc-Gly-OPFP (6)

This compound was prepared from Fmoc-Gly-OH and pentafluorophenol similarly to **5**. Yield 61.3%; m.p. 156.3–157.4 °C; $[\alpha]_{20}^{D}$ −1.3 (*c* 1, CH_2Cl_2); ^{1}H-NMR ($CDCl_3$): δ 7.82 (d, 2H, arom. Fmoc), 7.65 (d, 2H, arom. Fmoc), 7.47-7.25 (m, 4H, arom. Fmoc), 5.40 (t, 1H, NH), 4.54-4.42 (m, 5H, CH_2 Fmoc, CH Fmoc, $2H_\alpha$ Gly); ^{13}C-NMR ($CDCl_3$): δ 167.14 (CO ester Gly), 156.84 (CO urethane), 144.31, 142.00, 128.46, 127.77, 125.66, 120.70 (C arom. Fmoc), 68.14 (CH_2 Fmoc), 47.73 (CH Fmoc), 42.87 (C_α Gly); ^{19}F-NMR ($CDCl_3$) δ −152.44 (2F), −157.25 (1F), −161.96 (2F).

7. Synthesis of monomers

7.1. N-(N′-Acrylamido)butyramido-tbutyl-(tBu)-tyrosinyl ester (7)

To a stirred mixture of Tyr(*t*Bu)-*t*Bu (0.065 g, 0.2 mmol) in CH_2Cl_2 (5 mL) were added DIEA to obtain pH 8, *N′*-acrylamido-hydroxysuccinimidyl-butyric ester **9** (0.05 g, 0.2 mmol), HOBT (0.003 g, 0.02 mmol) and stirred overnight at 25 °C. The solution was then concentrated under reduce pressure and the residue purified by column chromatography on silica gel using ethyl acetate as eluent to give **7** (0.074 g, 87%); m.p. 114.8–115.7 °C; $[\alpha]_{20}^{D}$ +38.9 (*c* 1, CH_2Cl_2); ^{1}H-NMR ($CDCl_3$): δ 7.10 (d, 2H, arom. Tyr), 6.91 (m, 3H, arom. Tyr, NH Tyr), 6.71 (d, 1H, NH), 6.26-6.16 (m, 2H, 1H vinylic CH_2, vinylic CH), 5.63 (dd, 1H, 1H vinylic CH_2), 4.71 (m, 1H, H_α Tyr), 3.34 (dd, 2H, $2H_\beta$), 3.04 (m, 2H, CH_2–NH), 2.27 (t, 2H, CH_2–CO), 1.84 (m, 2H, CH_2); ^{13}C-NMR ($CDCl_3$): δ 172.99, 171.5, 166.55 (3 CO), 154.97 (C_β Tyr), 131.72, 131.70, 130.50, 126.75, 124.75 (C arom. Tyr, vinylic CH_2, vinylic CH), 82.95, 79.07, 29.5, 28.61 (2 *tert*-butyl Tyr), 54.40 (C_α Tyr), 38.11 (CH_2–NH), 34.50 (CH_2–CO), 25.62 (CH_2); MS (FAB): *m/z* 433 $[M+H]^+$, 455 $[M+Na]^+$, 865 $[2M+H]^+$, 889 $[2M+Na]^+$; Anal. $C_{24}H_{36}N_2O_5$ (C, H, N).

7.2. Tris(acetoxymethyl)-acrylamidomethane (8)

Tris(hydroxymethyl) acrylamido methane (5 g, 28.6 mmol) was added slowly in pyridine–acetic anhydride (50:50, v/v) (100 mL) at 0 °C. The stirring was pursued

overnight at r.t. The organic phase was then washed with 1 N HCl (3 × 10 mL), satured aq. NaHCO3 (2 × 10 mL) and water (2 × 10 mL). After drying on Na_2SO_4 and removal the solvent in vacuo, the residue crystallized in ethyl acetate/heptane (7/3 v/v) to give **8** (7.52 g, 87.3%); m.p. 96.5–97.4 °C; ^{1}H-NMR ($CDCl_3$): δ 6.34-6.06 (m, 3H, 1H vinylic CH_2, vinylic CH, NH), 5.72 (d, 1H, vinylic CH), 4.53 (s, 6H, 3 CH_2), 2.14 (s, 9H, 3 CH_3); ^{13}C-NMR ($CDCl_3$): δ 171.34 and 166.14 (CO ester and CO amide), 131.47 (vinylic CH), 127.85 (vinylic CH_2), 63.43 (3 CH_2), 59.00 (C), 21.42 (CH_3); Anal. $C_{13}H_{19}NO_7$ (C, H, N).

7.3. N^{γ}-Acrylamido-hydroxysuccinimidyl-butyric ester (9)

Hydroxysuccinimide (0.73 g, 6.37 mmol) was added to a solution of N^{γ}-acrylamidobutyric acid (1 g, 6.37 mmol) and DCC (1.57 g, 7.64 mmol) in methylene chloride. The mixture was stirred for 15 h at r.t. and the precipitated DCU was filtered. The filtrate was concentrated under reduce pressure and the residue purified by column chromatography on silica gel (eluent: EtOAc–hexane, 90:10, v/v) to give **9** (0.76 g, 47%): m.p. 93.5–95.4 °C; ^{1}H-NMR ($CDCl_3$): δ 6.29 (m, 2H, vinylic H and NH), 6.12 (dd, 1H, vinylic CH_2), 5.64 (dd, 1H, vinylic CH_2), 3.45 (q, 2H, $2H_{\alpha}$), 2.86 (s, 4H, 4H succinimidyl), 2.68 (m, 2H, $2H_{\beta}$), 2.05 (t, 2H, $2H_{\gamma}$); ^{13}C-NMR ($CDCl_3$): δ 193.00 (CO ester), 191.83; 188.89 (3 CO–N), 144.72 (vinylic CH_2), 139.65 (vinylic CH), 29.34 (2 C succinimidyl), 17.29 (C_{α}), 13.4 (C_{γ}), 12.00 (C_{β}); MS (FAB): *m/z* 255 $[M+H]^+$, 277 $[M+Na]^+$, 509 $[2M+H]^+$, 531 $[2M+Na]^+$; Anal. $C_{11}H_{14}N_2O_5$ (C, H, N).

8. Synthesis of telomeric substrates 11a, 12a, 13a

8.1. General procedure

Monomers **7**, **8**, **9** and/or **10** were reacted in anhydrous DMF (2 mL) (or THF). The different ratio of different monomers used for the synthesis of telomers are indicated with the yield of reactions in Table I. Different amounts of octanethiol telogen ($1/Ro$ = [Monomer]/[Telogen]) as indicated in Table I in the presence of AIBN ([AIBN] = [Telogen]/2) were then added and the mixture refluxed for at least 12 h until the complete disappearance of **7**, **8**, **9** or **10** (monitored by TLC and detected to UV light). At about the middle of the reaction, further addition of AIBN ([Telogen]/2) was effected. When all the starting monomers were reacted, the solution was concentrated under vacuum and the mixture was fractionated with a Sephadex LH20 column (2.5 × 35 cm, eluent: CH_2Cl_2–MeOH, 50:50, v/v) and the desired fractions (detected on TLC by charring with a methanol–sulfuric acid solution) were evaporated under reduce pressure to give **11a**, **12a**, **13a**. The DPn was specified by ^{1}H-NMR in CD_3OD by comparing the area of the signal due to the terminal methyl group of the telomer (distinct triplet; δ 0.9) with the area of the signal ascribed to aromatic protons of **7** (doublet; δ 6.9), to methylene protons of **8** (singlet; δ 4.3), to succinimidyl protons of **9** (singlet; δ 2.9) or to aromatic proton of **10** (doublet; δ 8.1).

8.2. Deprotection of cotelomer 11a

0.1 g of **11a** was stirred 24 h in 10 mL of a trifluoroacetic acid–methylene chloride mixture (3:7, v/v). The solution was then concentrated under vacuum until TFA was evaporated 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 G50 column (2.5 × 80 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 (0.079 g, 82%).

8.3. RGDSK coupling (to 12a and 13a)

Example: coupling of RGDSK to **12a**.

After deprotection of the benzyloxycarbonyl group of compound **4** (0.143 g, 0.126 mmol) by catalytic hydrogenolysis on Pd/C, the resulting deprotected peptide **4a** (0.126 g, 0.126 mmol) was added to a solution of telomer **12a** in methylene chloride (0.1 g, 0.0105 mmol) and the mixture was stirred 24 h in the presence of DIEA (pH 8–9). The mixture was concentrated under reduce pressure and the residue purified by Sephadex LH20 column (2.5 × 35 cm, eluent: CH_2Cl_2–MeOH, 50:50, v/v). Then, the desired fractions (detected on TLC by charring with a methanol–sulfuric acid solution) were evaporated under reduce pressure. At this point of the synthesis, the coupling percentage of the peptidic sequence was specified by comparing in ^{1}H-NMR peaks area assigned to the aromatic Mtr proton (6.6 ppm) to the terminal methyl signal in the hydrocarbon tail of the telomer (0.9 ppm). It is here noteworthy that for each telomer **12** and **13**, the NMR structural evaluation showed that the proportion of RGD moieties was similar to the succinimidyl groups one in the precursor telomers **12a** or **13a**. Finally, the hydrolysis of both *tert*-butyl and Mtr protective groups, was performed using a trifluoroacetic acid–methylene chloride–methyl phenyl sulfide mixture (3/5/2 v/v/v) at r.t. for 24 h. The solution was then evaporated under vacuum and precipitated in diethyl ether until trifluoroacetic acid was evaporated. The mixture was fractionated with a Sephadex G50 column (2.5 × 35 cm) and the desired fractions (detected on TLC

by charring with a methanol–sulfuric acid solution) were lyophilized to give a set of telomers **12** as a white powder (0.103 g, 70%).

9. Pharmacological assays

9.1. B16 cells culture

The murine B16 melanoma cells were obtained from ICIG (Villejuif, France). Stock cell cultures were maintained as monolayers in 75 cm^2 culture flasks in Glutamax™ (Eagle's minimum essential medium, Gibco, Paisly, Scotland) supplemented with 10% foetal calf serum (Bio West, Nuaille, France), 100 × vitamin solution (Gibco), 100 mM sodium pyruvate (Gibco), 100 × non essential amino acids (Gibco) and gentamycin sulfate (Gibco).

9.2. B16 cells cytotoxicty assay

For the cytotoxicity assay, B16 cells were plated into 60 mm Petri dishes (150 cells/dish) and allowed to adhere for 20 h before treatment. Then, medium was removed and replaced by new medium containing increasing concentrations of **11** and **13** from 0.01 to 1 μM and the cells were grown for 10 days at 37 °C in a CO_2 incubator. After this time, the 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. Colonies of more than 50 cells were counted. The surviving fraction was calculated as the ratio of coloning efficiencies of treated and untreated cells. The antiproliferative activity of the drugs, expressed as IC_{50}, corresponds to the drug concentration giving a 50% coloning efficiency compared with untreated cells [42,43].

9.3. In vivo antitumoural assay

For antitumoural assay, 5×10^5 B16 cells were subcutaneously implanted on the dorsum of 7 weeks male C57Bl6/Jco mice (Iffa Credo, L'Arbresle, France) on day 0. Animals were randomly assigned into two groups of six mice. On days 1, 5 and 9, treated mice received compounds **11** or **13** (5 μmol equivalent AraC kg^{-1}, corresponding to 10% of the LD50) by i.v. route (tail vein). Control animals were treated with solvent (NaCl 0.9%). Median survival times were determined for the respective groups. Antitumour activity is expressed as an oncostatic index: T/C × 100 [44].

10. Biodisposition studies

10.1. Radioactive labelling

Molecules were labelled with ^{125}I on the tyrosine moiety using the chloramine T method [45]. Purification of the labelled compounds was performed by ion exchange HPLC on anionic column Poros HQM, using a Biocad Sprint apparatus (Perseptive Biosystems). Specific radioactivities were respectively 125 μCi mg^{-1} for **11** and **12** and 135 μCi mg^{-1} for **13**.

10.2. Animal experiments

C57Bl6 mice were inoculated with 10^6 B16 cells by dorsal subcutaneous injection. Animals were used when the tumour diameter reached around 0.5 cm, then they received by intravenous injection 20 μCi of labelled compound dissolved in 100 μL of NaCl 0.9%. Mice were sacrificed at 15 min, 1 and 6 h post administration of the drug and immediately frozen in liquid nitrogen. Afterward, the whole body was sliced (40 μm) using a cryomicrotome. Tissue radioactive concentration was determined with a computer-controlled multi-wire proportional counter (Ambis 4000, B. Braun Scencetec). The number of particles per time and surface unit obtained with the gaseous detector is a linear function of the radioisotope concentration. Counts are recorded in 1.064.448 discrete detection points from which the composite image is displayed on a high resolution color monitor so that regions of interest may be directly evaluated and radioactive concentration can be expressed as percentage of the administered dose per g of tissue [46].

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