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# Synthesis and Preliminary Biological Assessments of RGD Bearing Biocompatible Telomers

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**Abstract**—Work reported herein deals with the synthesis and preliminary biological assessments of a new class of biocompatible telomeric carriers bearing peptidic RGDSK sequences as tumor cell targeting and tyrosine moieties labelled with $^{125}I$ as in vivo probe. The radioactivity levels obtained in several tissues, after the intravenous injections of these telomers in mice bearing grafted B16 syngenic melanoma showed that the addition of a RGD residue to a telomeric structure confers it an increased affinity for the highly vascularized zone surrounding the tumor. 

The preparation of drug bearing macromolecular conjugates in which therapeutic molecules are linked to a polymeric backbone by means of biodegradable covalent bonds has been widely investigated in recent years.[1–4] In this field, we have developed researches on the biomedical potentialities of a new class of low macromolecular delivery systems called 'telomers'.[5] These compounds are obtained by free radical polymerization of an acryloyl monomer derived from tris(hydroxymethyl)acrylamidomethane THAM **1** in the presence of an alkane or a perfluoroalkanethiol as a transfer reagent. Most of 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. Preliminary biological studies have shown that these multifunctional molecules exhibit good biocompatibility making them suitable for drug delivery systems.[6] It is noteworthy that the biodistribution in rat and mouse of THAM derived telomers, specified by whole body autoradiography, is quite ubiquitous in all tissues.[6] The anchorage of various antitumor 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.[7,8] Now, it seems necessary to ensure a specific tumor cell targeting to these prodrugs in order to reduce the toxicity of the active principle on normal cells. Work reported herein deals with the synthesis and preliminary biological assessments of telomeric delivery systems allowing the selective targeting of proliferative angiogenic vascular cells that support tumors growth.

Among the numerous surface receptors involved in a pathologic process like tumor metastasis, integrins, a class of heterodimeric glycoproteins, provided potential targets for directing chemotherapeutic agents to tumorigenic lesions.[9] In this field, the $\alpha v\beta_3$ integrin was reported to play a crucial role in the angiogenesis which is important for the development of metastatic colonies.[9] Many integrins can be inhibited by antibodies or small peptides that possess the RGD (Arg-Gly-Asp) sequence as a common receptor recognition motif. Grafting this sequence onto the backbone of macromolecular prodrugs could result in highly potent and selective inhibitors of integrins.

RGD peptides and small RGD peptidomimetics have received considerable attention.[10,11] Studies of a variety of linear and cyclic RGD peptides showed that additional amino acids modulate the activity of the RGD-containing sequence toward the $\alpha v\beta_3$ receptor. For instance, hydrophobic amino acids following the aspartic

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acid moiety (i.e., in position 4) increase it.[12] However, the hydrophilic amino acid serine also increases the inhibitory activity for the $\alpha v\beta_3$ receptor.[13] By contrast neither hydrophilic nor hydrophobic substitutions in position 5 seem to influence this activity.[14]

Considering all these data and to provide a good integrins accessibility for the RGD moieties, we chose to graft a peptidic chain containing the RGDSK sequence to the telomer backbone through a γ-aminobutanoic acid spacer. Furthermore, in order to allow the in vivo scintigraphic follow-up of the macromolecule, a tyrosine polymerizable monomer was used during the preparation of THAM derived cotelomers.

## Synthesis

Peracetylated THAM monomer **1** was easily synthesized from tris(hydroxymethyl)acrylamidomethane by using the conventional reactants acetic anhydride in pyridine at room temperature for 12 h. Compound **1** was obtained after recrystallization in an ethyl acetate/hexane mixture as a white powder (87% yield, F = 97.5 °C).

Concerning the preparation of the tyrosine derived monomer, *N*-γ-acrylamidobutyric acid[7] was first activated as its hydroxysuccinimidyl ester **2**. Consecutive coupling of this active ester to *O*-(*t*-butyl) *t*-butyl L-tyrosinate was performed in methylene chloride at room temperature and provided monomer **3** (F = 116 °C, 87% yield). These three monomers gave satisfactory elemental analysis and were fully characterized by NMR spectroscopy.

RGDSK **4** was prepared following conventional methods of liquid-phase synthesis applying fmoc-strategy (Fig. 1). 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 (2,3,6-trimethyl-4-methoxy-benzenesulfonyl moiety) in the case of arginine. Compounds **4** and **5** were obtained with the aid of BOP and TBTU coupling reagents respectively using TEA and DIEA as bases in methylene chloride. Pentafluorophenol (PFP) and DCC were both used to carry out the synthesis of synthons **6** and **8**. Following this pathway, all compounds gave satisfactory elemental analysis and were fully characterized by NMR spectroscopy and FAB-mass spectrometry. The RGDSK protected sequence was prepared with an overall yield of 43%.

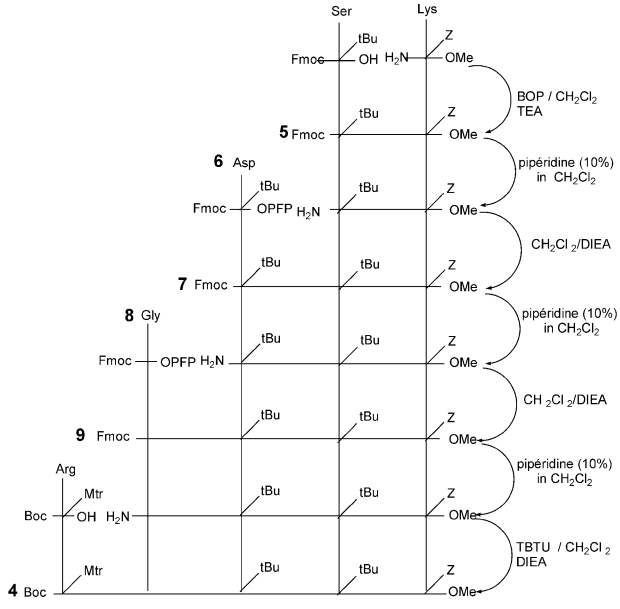


**Figure 1.** Synthesis of RGDSK sequence.

Telomerization experiments (Fig. 2) were carried out by refluxing THF under a nitrogen atmosphere in the presence of octanethiol as transfer reagent and AIBN as radical initiator. The AIBN concentration was roughly 10 times lower than the telogen's. The number average degree of polymerization (DPn), equal to the amount of repeating units (x + y + z), depends on the (telogen)/(monomers) ratio adjusted through both starting materials and experimental conditions.[15] The proportions of monomers **1**, **2** and **3** and octanethiol used are reported in Table 1. Each experiment was continued until the complete disappearance of the monomers. Telomers were purified by chromatography through a Sephadex LH20 column.

The structures of these cotelomers, that is the relative proportions of each tyrosine (x), activated butanoic acid (y) and THAM (z) moiety in the cotelomer and the DPn of the macromolecule, were determined in $^1$H NMR.

The relative proportions of each tyrosine (x), active ester (y) and peracetylated THAM (z) moiety in the cotelomer **11** were specified by comparing the peaks area assigned to the terminal methyl signal in the hydrocarbon tail (δ 0.9 ppm, 3H) to aromatic tyrosine protons (δ 6.79 ppm, 2xH), to succinimidyl protons (δ 2.61 ppm, 4yH) and to methylene protons of peracetytaled THAM (δ 4.30 ppm, 6zH).

The benzyloxycarbonyl protection of the side-chain amino group of lysine in RGDSK sequence **4** was removed by catalytic hydrogenation on Pd/C. Consecutive coupling of this peptide **10** to the active ester residues of the telomer **11** was carried out in methylene chloride at room temperature for 24 h under nitrogen atmosphere. The proportion of RGD sequences grafted onto the telomer was specified by comparing in $^1$H NMR peaks area assigned to the aromatic Mtr proton (δ 6.56 ppm, 1yH) to the terminal methyl signal in the hydrocarbon tail. It is noteworthy that for each telomer (**Tel H** and **Tel Ac**), the NMR structural evaluation showed that the proportion of RGD moieties was similar to the succinimidyl groups one in the precursor telomer **11**. The hydrolysis of both *t*-butyl and Mtr protective groups by using a trifluoroacetic acid/methylene chloride mixture (3/7 v/v) at room temperature for 24 h was checked by $^1$H NMR spectrum after a purification step and led to the compound **Tel Ac**. A consecutive

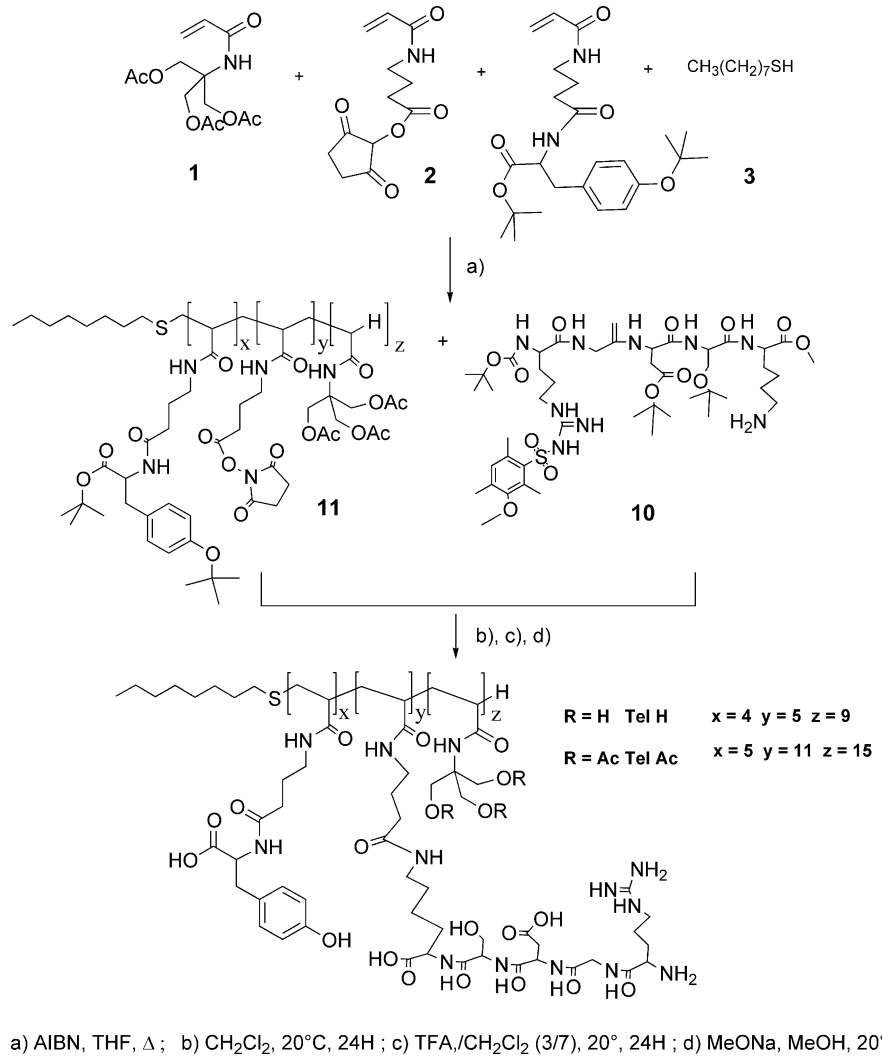


**Figure 2.** Synthesis of cotelomers **Tel H** and **Tel Ac**.

deacetylation of THAM residues by transesterification in methanol with a catalytic amount of sodium methylate was performed to obtain compound **Tel H**. Finally, **Tel H** and **Tel Ac** were submitted to a chromatography on Sephadex G25 column, then lyophilized.

## $^{125}$I-labelling of molecules

Each compound was dissolved in water at a concentration of 2 mg/mL. To 1 mL of solution was added 1 mCi of $^{125}$INa (IMS30 Amersham) then 100 μL of Chloramine T (10 mg/mL). After 5 min at room temperature, the reaction was stopped by addition of 100 μL of $Na_2SO_3$ (10 mg/mL). Labelled compound was purified by separation of non-reacted iodide by HPLC on anionic column (Poros HQ-M) using a NaCl gradient from 0 to 1 M. Labelling yield was around 60%.

## Animal experiments

C57Bl6 male mice, weighing 20–25 g and bearing grafted B16 syngenic melanoma tumors were given intravenously 20 μCi of each labelled compound dissolved in 100 μL NaCl 0.9%. 15 min, 1 h, and 6 h after administration, animals were sacrificed by ether inhalation. Tissue radioactive concentration (Table 2) was determined with a computer-controlled multi-wire proportional counter (Ambis 4000) allowing direct quantitation of regions of interest.[16]

**Table 1.** Physico-chemical data of cotelomers, **H** and **Ac**

| | Initial conditions [monomer]/[telogen] | | | Telomer structures | | | |
|---|---|---|---|---|---|---|---|
| Compd | **3** | **2** | **1** | x (Tyr) | y (OSu) | z (THAM) | DPn |
| **Tel H** | 8 | 3.2 | 8 | 4 | 5 | 9 | 18 |
| **Tel Ac** | 8 | 6 | 14 | 5 | 11 | 15 | 31 |

The concentrations of each monomer **1**, **2** or **3** used are expressed in relation to the octanethiol concentration.

## Results and Discussion

Table 2 shows radioactivity levels in several tissues as a function of time following administration. Both molecules

**Table 2.** Distribution of radioactivity in tissues after iv administration of 20μCi of labelled **Tel H** and **Tel Ac**

| Time after administration | 15 min | | 1 h | | 6 h | |
|---|---|---|---|---|---|---|
| Compd | **Tel H** | **Tel Ac** | **Tel H** | **Tel Ac** | **Tel H** | **Tel Ac** |
| Tumor | 2.04±0.74 | 1.74±0.65 | 0.81±0.18 | 2.14±0.59 | 0.30±0.09 | 1.44±0.27 |
| Stroma | 7.91±0.91 | 6.34±0.65 | 2.24±0.46 | 4.56±0.90 | 0.58±0.10 | 1.76±0.24 |
| Blood | 5.62±1.01 | 3.29±0.28 | 1.80±0.12 | 1.99±0.22 | 0.57±0.09 | 1.09±0.08 |
| Kidney | 21.2±4.51 | 31.4±5.26 | 19.5±3.21 | 18.5±3.19 | 10.8±2.24 | 8.01±1.54 |
| Liver | 2.36±0.58 | 2.11±0.35 | 1.01±0.32 | 0.94±0.17 | 0.34±0.12 | 0.52±0.12 |
| Thyroid | 18.5±1.87 | 8.57±1.15 | 78.5±12.5 | 54.5±10.2 | 97.1±17.3 | 75.4±15.2 |

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

exhibit 15 min after administration high concentration in kidney, indicating a fast urinary elimination.

At the tumor level, stroma was strongly labelled after 15 min and radioactivity spreads into tumoral tissue after 1 h. 6 h after administration, an homogenous radioactivity distribution was observed into the whole tumor.

These results also show that the acetylated molecule concentrates in the tissues more slowly than its non-acetylated homologue and that its elimination rate is slower. Particularly, concentration of the labelled compound in the tumor is significantly higher after 1 h for the acetylated molecule **Tel Ac**. The affinity of both compounds for the tumor stroma is demonstrated by the ratio tissue radioactivity/blood radioactivity $>1$ for all times following administration.

In conclusion, our results prove that the addition of a RGD residue to a telomeric structure leads to an increased affinity for the highly vascularized zone surrounding the tumor. On the other hand, the increase of lipophilicity involved by the acetylation reduces the elimination rate from the tumor. These properties can be used to conceive carriers able to specifically vectorize cytotoxic principles towards solid tumors. Work in this field is currently underway.

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