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Bioorganic & Medicinal Chemistry Letters
ELSEVIER
Bioorganic & Medicinal Chemistry Letters 14 (2004) 421–425

Inhibition of angiogenesis by THAM-derived cotelomers endowed with thalidomide moieties

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Received 16 July 2003; revised 1 October 2003; accepted 27 October 2003

Abstract—The synthesis of a tris(hydroxymethyl)acrylamidomethane (THAM)-derived cotelomer endowed with thalidomide units and a preliminary assessment of its biological activity are described. 4-Carboxy thalidomide and 4-(*N*-acryloyl) lysine thalidomide derivatives were prepared. The polymerization of these compounds with THAM in the presence of octanethiol as transfer reagent provided a water-soluble telomer bearing several thalidomide units. The ability of this telomer to inhibit angiogenesis in a mouse model of corneal neovascularization was compared to 4-carboxy thalidomide and thalidomide. A significant inhibition in area of neovascularization stimulated by a bFGF pellet was observed only in the mice treated with the telomer.

Thalidomide, a hypnosedative drug introduced in the 1950s, was first launched to prevent nausea in pregnant women. Its use was abruptly halted in 1961 with the discovery of its potential for causing severe birth defects in children whose mothers were exposed to the drug during the first trimester of pregnancy.[1–3]

However, numerous attractive properties of thalidomide have been observed since then. It was approved in 1998 by the Food and Drug Administration for the short-term treatment of cutaneous manifestations of moderate to severe erythema nodosum leprosum, a complication of leprosy (Hansen's disease).[4,5] Recently, it was described as a promising drug in the treatment of a number of cancers (in particular for multiple myeloma therapy)[1,6–8] and inflammatory- or immune-based complications of HIV infection and other diseases.[9]

In the field of medical oncology, one of its most promising properties is its antiangiogenic effect. It has been shown that thalidomide can inhibit angiogenesis induced by angiogenic cytokines such as basic fibroblast growth factor (bFGF) in the rabbit cornea and vascular endothelial growth factor (VEGF) in the corneas of mice.[10–12] Moreover for a few years, numerous metabolites or thalidomide derived compounds have been assayed to better understand the thalidomide activity and to specify their potentialities in medical oncology, notably as inhibitors of angiogenesis, TNF-α production or cyclooxygenase[13–16] or for the suppression of liver injury.[17]

In the course of our previous research, we focused on the phenomenon of angiogenesis, hypothesized 30 years ago to be an absolute requirement for the growth and metastasis of solid tumors.[18–20] We validated the therapeutic potential of oligomeric prodrugs of Ara-C called 'telomers' bearing RGD peptidic sequences known to be selectively recognized by specific receptors over-expressed on angiogenic sites.[21–23] Moreover, we obtained interesting biological results with telomeric carriers endowed with chemotherapeutics like Ara-C or 5-Fluorouracil[24,25] which prompted us to apply this concept of drug delivery to the design of macromolecular derivatives of thalidomide.

We predicted that the introduction of multiple thalidomide units on a telomeric carrier through an appropriate peptidic spacer arm would provide a macromolecule with antiangiogenic properties. Such a conjugate could

Keywords: Thalidomide; Telomers; Angiogenesis; Prodrug.
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doi:10.1016/j.bmcl.2003.10.045

be used for the specific delivery of antitumoral agents by the enhanced permeability and retention (EPR) effect.[26] In this paper, we describe the synthesis of a functionalized derivative of thalidomide (**1**) used for the preparation of a polymerizable monomer (**4**) bearing thalidomide grafted through a lysine spacer. The telomeric compound (**A**) resulting from the cotelomerization of this monomer (**4**) with tris(hydroxymethyl)acrylamido methane (**5**) (THAM) was evaluated as a potential inhibitor of angiogenesis on a chicken chorioallantoic membrane (CAM) assay and a mouse corneal micropocket assay.

1. Synthesis

The polymerization of THAM moieties needs the grafting of a suitable polymerizable functionality onto thalidomide which would not inhibit its biological activity. Towards that aim, we introduced a carboxy function in the 4-position of thalidomide aromatic group, such a localized modification should not modify the activity of this compound. The first stage of this synthesis was based on the condensation of *N*-*tert*-(butyloxycarbonyl) glutamic acid with trifluoroacetamide.[27] The acidolysis of the *tert*-(butyloxycarbonyl) protective group followed by the condensation of 1,2,4-benzenetricarboxilic anhydride, according to the technique proposed by Robin et al.,[28] led to acid thalidomide derivative **1** with an overall yield of 60% (Fig. 1).

In order to insure a maximum degree of freedom to the active principle grafted onto the polymer backbone, a lysine residue was introduced as a spacer arm during the preparation of the thalidomide derived monomer (Fig. 2). The synthesis of *N*-γ-acrylamido methyl lysinate was first achieved with 76% yield by coupling acryloyl chloride with *N*-α-*t*-butyloxycarbonyl methyl lysinate in methylene chloride at 0 °C in the presence of *N,N* diethylisopropylamine. The acid hydrolysis of the *tert*-butyloxycarbonyl protective group was then quantitatively performed by using a trifluoroacetic/methylene chloride (1/1) mixture-providing compound **2**. Pentafluorophenyl ester of 4-carboxy thalidomide was then prepared, with a yield of 78%, by condensing pentafluorophenol on compound **1** in dioxane. The reaction was carried out at room temperature in the presence of dicyclohexylcarbodiimide. Consecutive coupling of this active ester with *N*-γ-acryloyl methyl lysinate was performed in pyridine in the presence of catalytic amounts of diazabycyclooctane (DABCO) at room temperature, providing monomer **4** (F = 111 °C, 75% yield). This monomer gave satisfactory elemental analysis and was fully characterized by NMR spectroscopy and mass spectra.

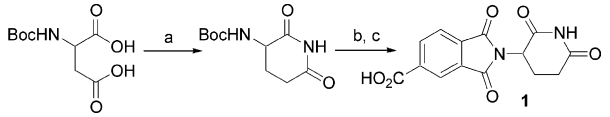

Figure 1. Synthesis of an analogue of thalidomide bearing an acid function in 4-position. (a) CF_3CONH_2, TEA, HOBT, EDC·Cl, CH_2Cl_2; (b) TFA/CH_2Cl_2; (c) 1,2,4-benzenetricarboxilic anhydride, TEA, THF, molecular sieves 4 Å.

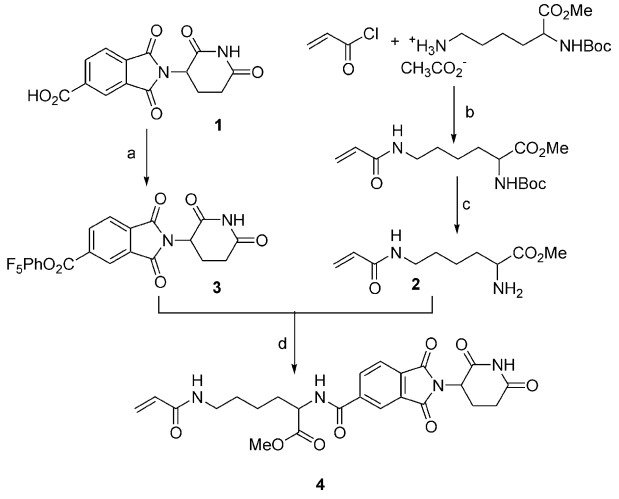

Figure 2. Synthesis of monomer **4** derived from thalidomide. (a) $HOPhF_5$, DCC, dioxane; (b) CH_2Cl_2, DIEA; (c) TFA/CH_2Cl_2; (d) Pyridine, DABCO.

The preparation and physico-chemical data of THAM co-monomer **5** were previously reported.[29]

Telomerization experiments (Fig. 3) were carried out by refluxing compounds **4** and **5** in methanol under a nitrogen atmosphere in the presence of octanethiol as transfer reagent and AIBN as radical initiator. The AIBN concentration was roughly ten times lower than that of the telogen. The number average degree of polymerization (DPn), equal to the amount of repeating units (x + y), depends on the (telogen)/(monomers) ratio adjusted through both starting materials and experimental conditions.[30]

The proportions of monomers **4** and **5** and octanethiol used are reported in Table 1. The telomerization was continued until the complete disappearance of the monomers.

Telomer **A** was purified by chromatography through a Sephadex LH20 column (eluent: ethanol/methylene

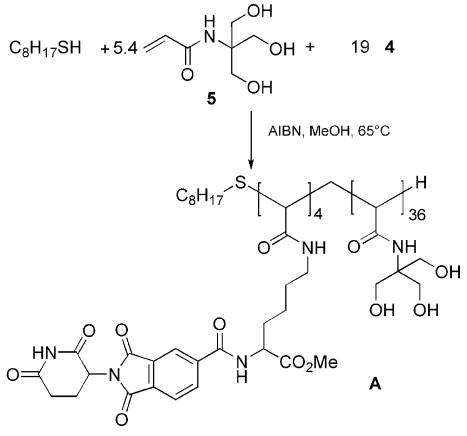

Figure 3. Synthesis of THAM derived telomeric carriers bearing thalidomide moieties.

chloride 1/1) and was obtained as a white powder (56% yield) fully soluble in water. The structure of this cotelomer, that is, the relative proportions of each THAM (x) and thalidomide residues (y) moiety in the cotelomer, and the DPn of the macromolecule were determined in ^{1}H NMR (solvent: DMSO-d_6) by comparing the peaks area assigned to the terminal methyl signal in the hydrocarbon tail (δ 0.9 ppm, 3H), to the thalidomide imide proton (δ 11.2 ppm, yH), and to methylene protons of THAM (δ 5.0 ppm, 6xH). The telomer **A** was composed of roughly 4 thalidomide units and 36 THAM moieties affording the water solubility to the macromolecule.

2. Biological assays

2.1. Bovine capillary endothelial (BCE)

Cell Proliferation Assay Bovine capillary endothelial cells were obtained and grown as previously described.[31] A cell suspension (30,000 cells/mL) was made in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% bovine calf serum (BCS) (Life Technologies, Inc.), 0.29 mg/mL L-glutamine, 100 units/mL penicillin and 100 μg/mL streptomycin (1% GPS) (Gibco), plated onto gelatinized 24-well culture plates (0.5 mL/well), and incubated (37 °C, 10% CO_2) for 24 h. The media was replaced with 0.25 mL of DMEM with 5% BCS and 1% GPS and the test sample was applied. Cells were challenged with thalidomide, compound **1** and telomer **A** (200 μM down to 0.02 μM). After 20 min of incubation, media and bFGF were added to obtain a final volume of 0.5 mL of DMEM with 5% BCS, 1% GPS, and 1 ng/mL bFGF. After 72 h, cells were dispersed in trypsin, resuspended in Hematall (Fisher Scientific, Pittsburgh, PA), and counted by Coulter counter.

2.2. Chick chorioallantoic membrane (CAM) assays

Chick chorioallantoic membrane (CAM) assays were performed as described.[32,33] Briefly, three-day-old fertilized white Leghorn eggs (Spafas, Norwich, CT) were cracked, and embryos with intact yolks were placed in 100 × 20 mm Petri dishes. After three days of incubation (37 °C and 3% CO_2), a carboxymethylcellulose (Fisher Scientific, Fair Lawn, NJ) disc containing thalidomide or telomer **A** (1 μg and 10 μg) was applied to the CAM of individual embryos.

After 48 h of incubation, embryos and CAMs were observed by stereomicroscope. The effects on the developing vasculature were recorded at 48 h after implantation of the 0.5% carboxymethylcellulose pellet containing various drugs.

2.3. Murine corneal neovascularization

Pellets containing basic fibroblast growth factor bFGF (80 ng/pellet) were made of the slow-release polymer Hydron (polyhydroxyethylmethacrylate [polyHEMA]) as previously described.[34]

Male C57 Bl/6 mice were anesthetized with 300 μL of 2.5% Avertin. The eyes were topically anesthetized with 0.5% proparacaine HCl (Ophthetic, Allergan). Using an operating microscope (Zeiss, Oberkochen, Germany), a central, intrastromal linear keratotomy (approximately 0.6 mm length) was performed with a 30° micro-knife (Xomed, Florida). The eye was then proptosed using forceps. Using a modified von Graefe knife no. 3 (2×30 mm), a micropocket was dissected toward the temporal limbus. For bFGF-containing pellets, the pocket was extended to 1.0 mm of the temporal limbus. A single pellet was placed on the corneal surface at the base of the pocket with jeweler's forceps, and, using one arm of the forceps, the pellet was advanced to the temporal end of the pocket. Antibiotic ointment (erythromycin) was applied once to the operated eye. The same procedure was repeated on the other eye. Control group was injected intraperitoneally (ip) daily with 250 μL 0.45% methyl cellulose. Treated groups were injected ip with 50 mg/kg daily equivalent dose of thalidomide. Each group consisted of both eyes of 6 mice.

The eyes were examined by slit lamp biomicroscopy on postoperative day 6 after pellet implantation. Mice were anesthetized with isofluorane and the maximum vessel length (VL) of the neovascularization zone, extending from the base of the limbal vascular plexus toward the pellet, was measured with a linear reticule through the slit lamp. The contiguous circumferential zone of neovascularization was measured as clock hour with a 3600 reticule (where 300 of arc equals 1 clock hour). The percentage of inhibition of vessel growth was calculated as the area of neovascularization in the treated mice (T)

Table 1. Physico-chemical data of cotelomer **A**. The concentrations of each monomer **4** or **5** used are expressed in relation to the octanethiol concentration

	Initial conditions [monomer]/[Telogen]		Telomer structures		
Comp	**4**	**5**	y (Thal)	z (THAM)	DPn
Tel A	5.4	19	4	36	40

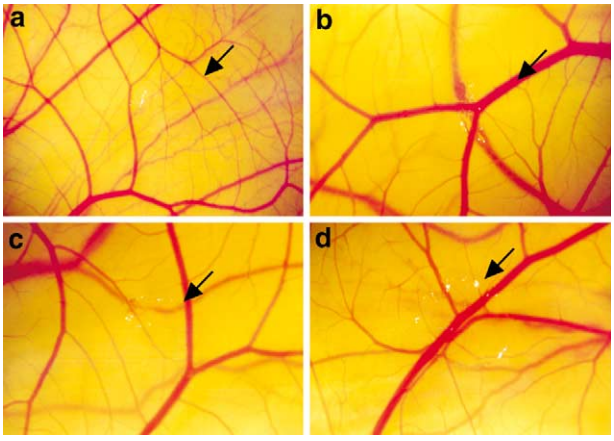

Figure 4. Representative CAM at day 4 after implantation of carboxymethylcellulose pellets as Control (a) or containing thalidomide (b), compound **1** (c) or telomer **A** (d). None of the compounds show significantly less neovascularization.

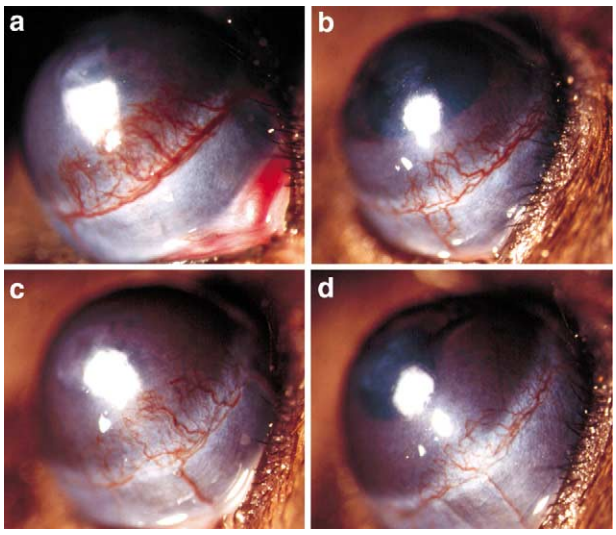

Figure 5. Representative corneas at day 6 after implantation of bFGF pellets from Control (a), thalidomide (b) compound **1** (c) and telomer **A** (d). (c) and (d) show markedly less neovascularization.

Table 2. Inhibition of bFGF-induced corneal neovascularization by thalidomide and analogues, expressed as percent of control on day 6

	PD	VL	CH	Area	Inhibition
Control	1	0.98±0.08	2.4±0.6	1.51	—
Thd	1	0.9±0.1	2.83±1	1.6	—
1	1	0.96±0.1	2.35±0.6	1.42	6%
Telo A	1	0.75±0.1	2.3±1.2	1.08	28%

divided by the area of neovascularization in the untreated control mice (C). The area is calculated as oval structure = Vessel Length (VL) × Clock Hour (CH) × Pellet Distance (PD) from the limbus × 0.2 × π.

3. Results and discussions

Initial investigations were performed on the CAM. Neither thalidomide nor any of the THAM-derived cotelomers endowed with thalidomide moieties exhibited any inhibitory activity on blood vessel growth (Fig. 4). Similarly, thalidomide, compound **1** and telomer **A** had no effect on bFGF-induced proliferation of endothelial cells in culture (results not shown). These results were expected as it has been proposed that thalidomide must be metabolized by the liver to form an epoxide that is the active teratogenic metabolite.[35]

Based on the requirement for liver metabolism, we tested thalidomide, acid thalidomide derivative **1** and telomer **A** on angiogenesis induced by bFGF in the mouse corneal micropocket model. A significant inhibition ($p < 0.05$, $n = 12$ eyes) in the area of neovascularization due to the bFGF pellet was seen only in mice treated with the telomer **A** (Fig. 5, Table 2). However, a very significant change in the architecture of the vessels was seen in mice treated with the acid thalidomide derivative **1**. Vessels looked very fragile and the density of vessel ingrowth in compound **1**-treated animals was markedly reduced. However, this fact is not taken into consideration when variants are put into formula for calculating the area. Due to the lack of an objective grading scale, these results are not presented. Thalidomide did not inhibit corneal neovascularization at the dose of 50 mg/kg. However, inhibition was seen at a teratogenic dose (200 mg/kg) of thalidomide.[11] No treated animal demonstrated any signs of sedation, toxicity or weight loss.

In summary, we have shown that acid thalidomide derivative **1** and telomer **A** were more potent than thalidomide at equivalent doses in an in vivo angiogenesis assay. Evaluation of the in vivo antitumor activity of these compounds is warranted.

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