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Preliminary biological evaluations of new thalidomide analogues for multiple sclerosis application

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ABSTRACT

The present work deals with the synthesis of a new series of thalidomide derivatives for therapeutic applications. These compounds were evaluated in vitro on a human endothelial cell line EA.hy926 for their antiproliferative potential and in vivo on an experimental animal multiple sclerosis model called EAE as angiogenesis inhibitors. The preliminary results obtained on EAE assays seem to validate that anti-angiogenesis compounds could be promising tools for the treatment of MS.

Forty years ago J. Folkman hypothesized that angiogenesis, the sprouting of new blood vessels from pre-existing ones, was a prerequisite phenomenon for a tumor's supply in nutrients and oxygen.[1] Since then, various anti-angiogenesis strategies have been investigated against tumor development. This extensive research led to the recent FDA approval of the first anti-angiogenic agent bevacizumab (Avastin, Genentech), a humanized anti-VEGF antibody, used in combination with chemotherapy for the treatment of metastatic colorectal cancer.[2] In the same field, vascular targeting strategies are currently being explored as promising tools for a selective shutdown of any established tumor vasculature, followed by rapid tumor cell death.[3,4]

In the course of our previous research, we focused on angiogenesis for oncological applications. We validated the therapeutic interest of oligomeric prodrugs called 'telomers' bearing Ara-C and endowed with RGD peptidic sequences for selective delivery of the drug on angiogenic sites.[5] Following these studies on angiogenesis targeting and/or inhibition, we explored the anti-angiogenesis potential of thalidomide, when grafted on a telomeric backbone. Indeed, thalidomide is a 're-discovered' drug which shows significant anti-angiogenic activity.[6] Thus, our introduction of multiple thalidomide units on a telomeric carrier led to a macromolecule exhibiting a significant inhibition of corneal neovascularization in a mouse model assay.[7]

Besides cancer therapy, other studies have revealed that pathological angiogenesis occurs in a number of relevant diseases, such as blinding ocular disorders,[8] or chronic inflammatory diseases like rheumatoid arthritis, atherosclerosis or psoriasis.[9]

Recently, angiogenesis has been implicated in the inflammatory phase of multiple sclerosis (MS), a chronic autoimmune inflammatory disease, and it may represent a target for therapeutic intervention.[10,11] Today the overall mechanisms by which thalidomide exerts its anti-tumor activity still remain to be elucidated, but thalidomide reduces the levels of angiogenic factors including TNF-α, VEGF and IL-6.[12] Aside from its role in angiogenesis, TNF-α is an inflammatory mediator also implicated in the pathogenesis of cerebral endothelial damage in MS.[13] The anti-angiogenic effect of thalidomide and its negative feedback on TNF-α production prompted us to imagine new functionalized derivatives of thalidomide, some of which could subsequently be grafted on amphiphilic molecular carriers, for intervention in MS.

In the present work, we have undertaken several structural development studies and obtained a series of thalidomide analogues substituted on the phthalimide ring system (Scheme 1). Some, like 3- or 4-nitro thalidomide,[14,15] have been used as starting materials for the preparation of new amino-thalidomide derivatives. Considering the hydrophobic nature of thalidomide, we also investigated the biological impact of derivatives containing a

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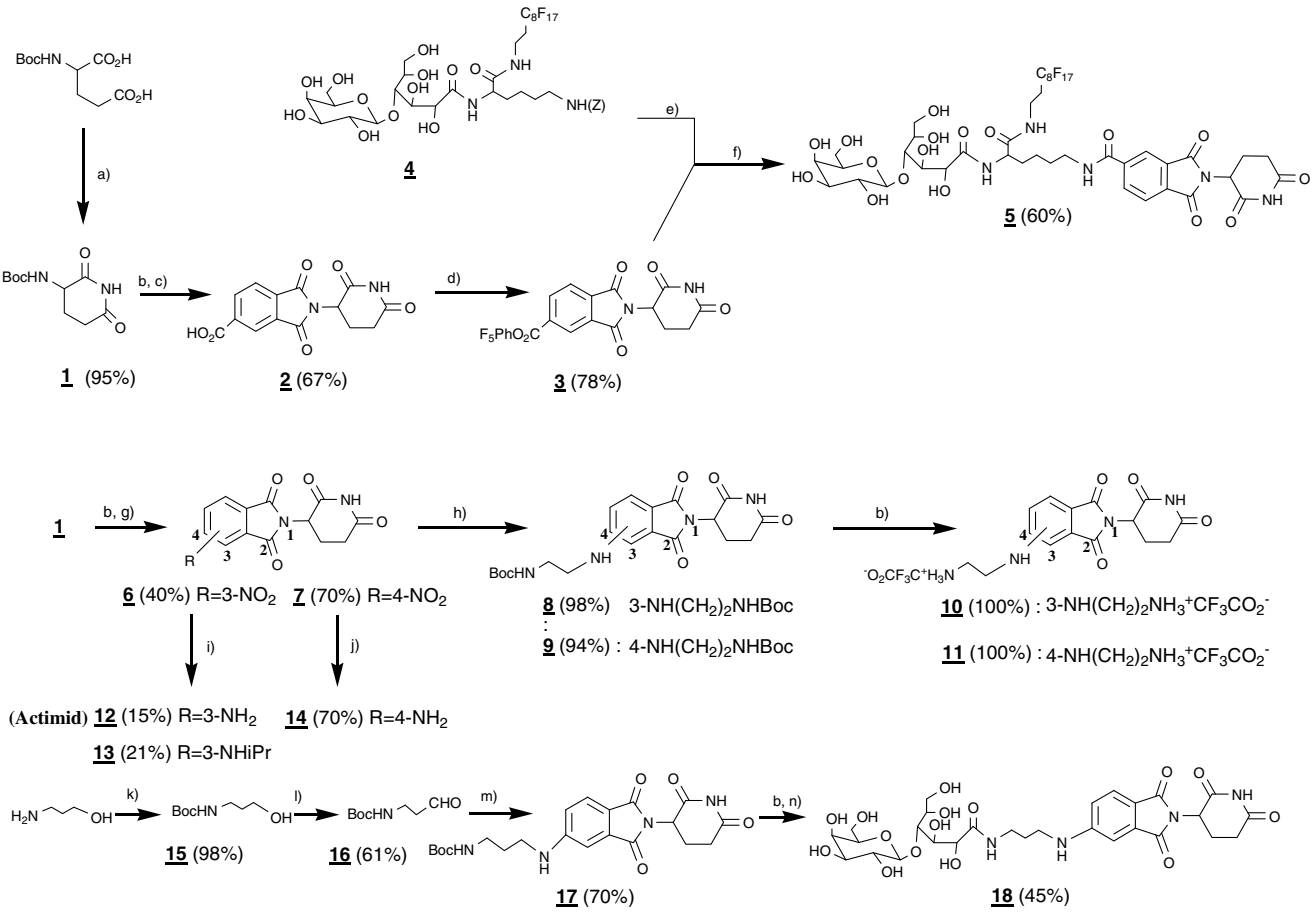

Scheme 1. Synthesis of thalidomide analogues. Reagents and conditions: (a) CF_3CONH_2, TEA, HOBT, EDC·Cl, rt; (b) CF_3CO_2H/CH_2Cl_2 3:7, 0 °C to rt; (c) 4-carboxy phthalic anhydride, TEA, THF, reflux, molecular sieves 4 Å; (d) $HOPhF_5$, DCC, dioxane, rt; (e) H_2/Pd/C, EtOH, rt; (f) DIEA, MeOH, rt; (g) 4-nitro or 3-nitro phthalic anhydrides, AcOH, reflux, molecular sieves 4 Å; (h) $BocNHCH_2CHO$, H_2/Pd/C, THF/DMF (9:1), rt; (i) H_2/Pd/C, acetone, rt; (j) H_2/Pd/C, AcOH glacial, rt; (k) BocON, TEA, CH_2Cl_2, rt; (l) PCC, CH_2Cl_2, célite, rt; (m) **7**, H_2/Pd/C, DMF/THF (9:1) rt; (n) lactobionolactone, TEA, 2-methoxyethanol, 60 °C.

hydrophilic moiety, such as lactobionamide (compound **18**), or grafting on a fluorinated amphiphilic carrier, which we have previously examined (compound **5**).[16] These new amphiphilic thalidomide derivatives should exhibit an increased bioavailability and a better therapeutic index than the parent drug.

The anti-angiogenic activities of 4-carboxy thalidomide **2**, and compounds **6**–**11** were evaluated in vitro through their inhibitory effect on the proliferation of a human endothelial cell line EA.hy926. Amphiphilic compounds **18** and **5**, and some of their intermediate analogues **2**, **11**, and **14** were screened in vivo on one of the best-characterized experimental animal model for MS study, namely the experimental autoimmune encephalomyelitis (EAE) model.[17] All the compounds were prepared by usual organic synthesis methods, as shown in Scheme 1, and gave appropriate analytical values.

Compounds **1**, **2**, **6**, and **7** were obtained, as previously described,[7] from (Boc)-aminoglutarimide **1** upon phthalylation, with phthalic anhydride derivatives: 4-carboxy, 3-nitro or 4-nitro phthalic anhydride. The pentafluorophenyl ester of 4-carboxy thalidomide (compound **3**) was then obtained by condensation of pentafluorophenol to compound **2** in dioxane. Consecutive coupling of **3** to the amphiphilic carrier **4** (prepared following our published synthetic route)[16] provided compound **5** in good yields.[18]

4-Nitro thalidomide **7** was reduced under catalytic hydrogenation in glacial acetic acid to produce the 4-amino derivative **14**, in quantitative yield. Surprising, the same reaction when applied to the 3-nitro thalidomide **6** in acetone as described by Muller et al.,[19] led to a mixture of the expected 3-amino-thalidomide or 'Actimid' **12** and of (*N*-isopropyl)-amino thalidomide **13** in low yields. We assumed that in this last case the amino group formed under these reductive conditions partially reacted with acetone through a reductive amination to give **13**. In an attempt to verify this hypothesis and take advantage of this side reaction for the preparation of new amino-thalidomide derivatives, we investigated the condensation of commercial (BOC)-aminoethanal on nitro-thalidomide under the same reductive conditions, using THF as solvent. This reaction provided the *N*-(aminoethyl)-amino derivatives **10** and **11** in good yields after hydrolysis of their corresponding *tert*-butyloxycarbonyl protective groups. Due to the poor chemical stability of previous (BOC)-aminoethanal, we lastly chose to perform the same reaction with (BOC)-aminopropanal **16** obtained in two steps from aminopropanol. The reductive amination of 4-nitro thalidomide in the presence of compound **16**, then provided compound **17**. The amphiphilic amino thalidomide analogue **18** was prepared after acidic deprotection and subsequent condensation of **17** with lactobionolactone.[18]

Although thalidomide is a prodrug requiring metabolic activation to exert an anti-angiogenic effect, it spontaneously hydrolyzes at physiological pH to give a cascade of hydrolysis products.[20] Some of them, as well as thalidomide, showed an in vitro inhibitory activity against endothelial cells without the need for prior metabolic activation.[15,21–23]

On the basis of these in vitro evaluations, some of our thalidomide analogues (compounds **2**, **6**–**11**) were first screened for their

ability to inhibit the proliferation of EA.hy926 cells, compared to thalidomide used as a standard. The hybridoma EA.hy926 is a fusion product between HUVEC and the epithelial cancer cell line A549.[24] This established cell line constitutes a valuable model to evaluate a potential effect on endothelial cells.[25] Moreover, its susceptibility to xenobiotics was recently shown to be comparable to that of HUVEC and this model could constitute an alternative material for routine cytotoxicity studies.[26] In vitro evaluations were performed on almost all thalidomide analogues except amphiphilic derivatives **5** and **18**, which were only used in the in vivo experiments.

All products were tested in aqueous solution containing 0.01% DMSO. In those conditions, all results are expressed comparatively to the control using solvent alone and no drug. To determine an IC_{50} value, a linearization of results is essential. Therefore, inhibition results were expressed as percent compared to control versus the logarithm of the concentration used. Inhibition studies on EA.hy926 are presented in Figure 1.

Five compounds (**11**, **7**, **9**, **10**, and **14**) exhibit an inhibitory activity on EA.hy926 cells. Except compound **14**, all present an activity higher than thalidomide and two of them, **11** and **7**, at a one magnitude lower dose. The amino derivative **14** and thalidomide show the same inhibiting activity of endothelial cell proliferation.

Extrapolation of the dose–response curves gives the IC_{50} value of each compound: thalidomide (equipotent to **14**) and **10** have, respectively, an IC_{50} around 200 and 150 μM, whereas an IC_{50} of 41 μM is found for derivative **9**. The most active products **11** and **7** significantly inhibit endothelial cells, respectively, at concentrations of 10 and 12 μM. It has to be noted that at these concentrations no activity on cellular growth was found for other tested products.

Some structure–activity relationships could be gained from these results. Introduction of a substituent in the C4 position of the aromatic ring seems to be required for inhibitory effect. In the case of compound **10**, we can speculate that the degree of freedom of the *N*-(aminoethyl)-amino group at the C3 position allows a productive receptor-mediated interaction with the targeted cells. However, among C4-substituted derivatives, 4-carboxy thalidomide derivative (compound **2**) exhibits surprisingly no inhibitory effect.

The influence of the electronic impact of the phthalimide ring substituents is not clear at this stage. Indeed, the two more active compounds **7** and **11**, respectively, substituted by an electron-withdrawing nitro group or by an electron-donating *N*-(aminoethyl)-amino group were equipotent in anti-angiogenic effect. A similar reversal of biological activity between derivatives with opposite electronic substituents was previously reported in the literature.[27]

In parallel to these preliminary in vitro assays, we investigated the potential of a series of C-4 substituted thalidomide analogues for MS utilization. For these in vivo experiments, we explored the combination of thalidomide to hydrophilic or amphiphilic ligands through the preparation of two new thalidomide derivatives: compounds **5** and **18**. These two amphiphilic compounds, derived either from 4-carboxy thalidomide **2** (for which we previously reported the anti-angiogenic behaviour[7] on a mouse corneal model) or from *N*-(aminopropyl)-amino thalidomide **17**, were evaluated on an experimental multiple sclerosis model and compared to some of their intermediates (compounds **2**, **11**, and **14**) and to free thalidomide.

The effectiveness of these compounds was tested in the MOG (35-55) peptide-induced experimental autoimmune encephalomyelitis (EAE) in C57BL/6J mice.[28] Six- to eight-week-old mice ($n = 34$) were immunized via a single flank injection of 200 μg of MOG (35–55) peptide in CFA with 10 mg/ml added *Mycobacterium tuberculosis* on day 0 and also received 200 ng pertussis toxin ip on days 0 and 2 post-immunization (pi). Animals were scored blindly as follows: 0, no change; 1, flaccid tail; 2, poor righting reflex; 3, one hind limb paralyzed; 4, both hind limbs paralyzed; and 5, moribund.

Dosing of thalidomide (100 mg/kg), thalidomide derivatives (100 mg/kg thalidomide equivalent) or vehicle (0.5% CMC) commenced on d7 pi and continued for up to 15 days. Figure 2 illustrates the clinical progression for vehicle and treated mice. The vehicle controls showed a gradual increase in mean clinical score starting on d13 pi reaching a maximum on the day of sacrifice (d22). Thalidomide treated mice also showed acute disease. Compound **14** showed about the same clinical progress as the control group. Surprisingly, the effects of compounds **5** and **11** were very similar and accelerated the development of acute EAE. We do not have data which would allow speculation on the mechanism for disease acceleration with these two compounds, but they were the only two that containing fluorinated side chains. 4-Carboxy derivative **2**, which was inert in vitro, produced diminished disease in this acute model and we also observed a decrease in clinical signs for amphiphilic *N*-(aminopropyl)-4-amino thalidomide derivative **18**. Thus, some compounds showed activity in vivo that was not predicted from in vitro experiments.

In conclusion, we report herein the synthesis of new thalidomide derivatives bearing either a carboxy or an aminoalkyl group on their phthalimide ring. The in vitro and/or in vivo biological activities of these compounds were evaluated. In vitro assays carried out on EA.hy926 cells do not allow us to propose constructive structure–activity relationships. Indeed, even if the C4 substitution seems to be a prerequisite to the antiproliferative effect, *N*-amino-

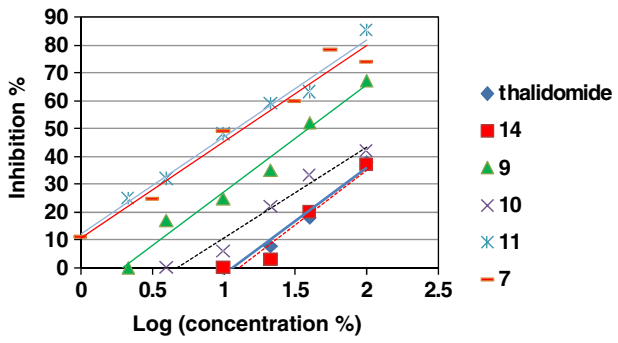

Figure 1. Effect of thalidomide derivatives on the proliferation of EA.hy926 cells.

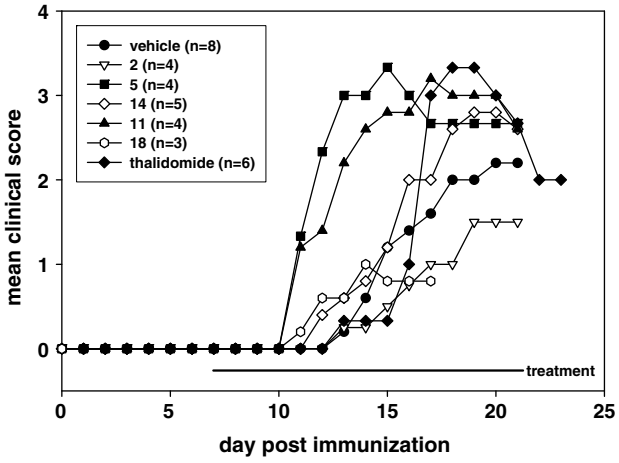

Figure 2. Mean clinical scores for EAE mice treated with vehicle, thalidomide and compounds **2**, **5**, **11**, **14**, and **18**.

propyl-3-amino derivative **10** shows a noticeable activity whereas 4-carboxy analogue **2** does not exhibit any effect. Such a negative result of compound **2** was previously observed on a bovine capillary endothelial cells model.[7] Nevertheless, this 4-carboxy derivative showed an in vivo activity. Indeed, the second part of this work focused on the effectiveness of our new thalidomide derivatives to interfere with the progression of EAE. A series of amphiphilic derivatives was evaluated and compared to free thalidomide. Whereas the parent drug does not exhibit any efficiency in decreasing the clinical signs of EAE when delivered in 0.5% CMC, 4-carboxy thalidomide **2** and amphiphilic thalidomide analogue **18** show decreased clinical signs in this model. As regards 4-carboxy derivative, it has to be underlined that its combination to a fluorinated amphiphilic carrier does not provide any positive effect. On the other hand, the linkage of a lactobionolactone moiety on *N*-(aminopropyl)-4-amino thalidomide **17**, providing amphiphilic compound **18**, led to a promising activity in EAE model.

These preliminary results validate that angiogenesis is an interesting target for the treatment of MS. Although all compounds were derived from thalidomide, only compounds **2** and **18** showed substantive in vivo activity and will be starting point for further investigations. Indeed currently, no clear structure–activity relationships can yet be drawn from these in vitro and in vivo assessments.

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18. Compound **5**: white powder (60%), mp 199.1–199.4 °C; $[\alpha]^{D}_{20}$ +1.3 (c: 0.25, DMSO), Anal. Calcd for $C_{42}H_{46}F_{17}N_5O_{17}\cdot 2H_2O$: C 40.28, H 3.99, N 5.59, found: C 40.56, H 4.14, N 5.56; MS (FAB+): [thalidomide]$^+$ = 257, [M+Na]$^+$ = 1238. ^{1}H NMR (250 MHz, DMSO-d_6, δ): 11.18 (1H, s), 8.45 (1H, d, J = 7.47 Hz), 8.32 (1H, s), 8.11 (1H, t), 8.07 (1H, d, J = 7.47 Hz), 7.71 (1H, m), 7.22 (1H, m), 5.47 (1H, m), 5.19–5.13 (2H, m), 4.98–4.80 (3H, m), 4.65–4.46 (4H, m), 4.36 (1H, d, J = 4.6 Hz), 4.29–3.85 (8H, m), 3.73–3.44 (8H, m), 2.44–2.10 (6H, m), 1.59–1.34 (6H, m). ^{13}C NMR (250 MHz, DMSO-d_6, δ): 174.28; 173.57; 173.24; 173.21; 170.0; 167.14; 164.64; 140.71; 134.47; 129.63; 124.05; 122.14; 105.27; 86.32; 76.19; 73.68; 73.38; 72.04; 71.77; 71.58; 68.59; 62.66; 60.24; 52.58; 49.59; 40.85; 33.81; 31.38 (CH_2Rf, t); 26.52; 24.94; 23.15; 22.39. ^{19}F NMR (250 MHz, DMSO-d_6, δ): −80.15; −113.36; −121.62; −122.42; −123.14; −125.68. Compound **18**: yellow powder (45%), mp 121.8 °C (dec); $[\alpha]^{D}_{20}$ +6.33 (c: 0.66, CD_3OD); MS (ESI) *m/z*: [M+H]$^+$ = 671, [M+NH_4]$^+$ = 688, [M+Na]$^+$ = 693, [M+K]$^+$ = 709. ^{1}H NMR (250 MHz, CD_3OD, δ): 7.51 (1H, d, J = 8.4 Hz), 6.94 (1H, s), 6.87 (1H, d, J = 8.4 Hz), 5.0 (1H, dd, J = 12.2 Hz), 4.52 (1H, d, J = 7.2 Hz), 4.29 (1H, m), 4.18 (1H, m), 3.82–3.27 (14H, m), 2.90–2.52 (3H, m), 2.07 (1H, m), 1.81 (2H, m). ^{13}C NMR (250 MHz, CD_3OD, δ): δ = 174.0; 173.3; 170.4; 168.4; 167.9; 154.6; 134.6; 124.8; 116.9; 115.4; 105.5; 104.4; 81.9; 75.8; 73.4; 72.6; 71.7; 71.4; 71.1; 69.0; 62.4; 61.3; 49.0; 40.0; 36.3; 30.8; 28.96; 28.2; 22.4.
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