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Dual bioactivity of resveratrol fatty alcohols: Differentiation of neural stem cells and modulation of neuroinflammation

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Abstract—The synthesis of resveratrol fatty alcohols (RFAs), a new class of small molecules presenting strong potential for the treatment of neurological diseases, is described. RFAs, hybrid compounds combining the resveratrol nucleus and ω-alkanol side chains, are able to modulate neuroinflammation and to induce differentiation of neural stem cells into mature neurons. Acting on neuroprotection and neuroregeneration, RFAs represent an innovative approach for the treatment or cure of neuropathies.

Neural cell death is one of the major characteristics of degenerative neuropathologies like Alzheimer's disease (AD) or Multiple sclerosis (MS), although loss of cell functions of neurons and/or glial cells is also known to contribute to these degenerative diseases.

At present, most treatments of these incurable neurological disorders are symptomatic. The discovery of the presence of neural stem/progenitor cells (NSCs) in the adult brain has opened new approaches for therapeutic interventions.[1] NSCs are multipotent progenitor cells which can differentiate into all different nervous cell types (e.g., neurons, astrocytes and oligodendrocytes) and can contribute to neurogenesis in adulthood.[2] The differentiation of these NSCs is regulated by key factors involved in neurogenesis and stem cell development. Hence, potent inducing agents for NSC differentiation represent an innovative approach with good potential to treat or even cure neurological diseases by resupplying the central nervous system (CNS) with new mature nerve cells. Some proteic growth factors [e.g., Fibroblast growth factor (FGF) and Epidermal growth factor (EGF)] are able to induce the differentiation of NSCs into mature neural cells.[3] However, few non-proteic synthetic molecules have been shown to confer such effect.[4] Recently, Schultz et al. showed that a class of 4-aminothiazoles can induce, in a dose-dependent manner, neuronal differentiation of adult hippocampal neural progenitor cells.[5]

A second factor contributing significantly to the cell death in patient's brain tissues is the inflammatory processes accompanying neurodegenerative diseases.[6] During the development of neurodegenerative or demyelinating diseases, microglial cells, the brain resident monocyte-macrophage cell population, become highly activated.[7] This activation produces large amounts of devastating pro-inflammatory cytokines, like tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β) as well as free radicals like nitric oxide (NO) and superoxide anion.[8,9] In AD, the extracellular depositions of amyloid beta (Aβ) represent the major histological lesion and are responsible for the death of neurons by a currently unclear mechanism. One theory involves neuroinflammation which is supported by studies showing clustering of microglial cells within Aβ depositions in human brain tissues.[10]

Previous studies have shown that *n*-hexacosanol, a long chain primary alcohol containing 26 carbon atoms, confers neurotrophic activity. It has been

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shown that the length of the chain and the ω-hydroxyl function are essential for the biological activity.[11] Besides, in order to take into account the presumed contribution of inflammatory and oxidative phenomena in neurodegenerative pathologies, we decided to synthesize small molecules bearing a ω-alkanol side chain combined with a neuroprotective moiety. Structure–activity relationship studies led to the identification of compounds, presenting even better neurotrophic activities with a shorter chain length. Moreover, some of these fatty alcohol derivatives were able to induce differentiation of neural stem cells into mature neurons[12,13] or to induce the differentiation of oligodendrocyte precursors into mature oligodendrocytes.[14]

In view of these encouraging findings, we focused on the elaboration of new hybrid compounds. These molecules combine the neuroregenerative activities of the ω-alkanol structure and the neuroprotective effects of an anti-inflammatory and anti-oxidative core, *trans*-resveratrol (*trans*-3,4′,5-trihydroxystilbene). Thus, long chain fatty alcohols bearing the *trans*-resveratrol as a neuroprotective core, the resveratrol fatty alcohols (RFAs), were prepared (Fig. 1). Resveratrol is a phytoalexin mainly found in plants, grapes and peanuts which has been shown to display a wide range of interesting and useful bioactivities (e.g., cancer chemoprotective, anti-inflammatory, anti-platelet aggregation, anti-oxidative and anti-bacterial activities).[15]

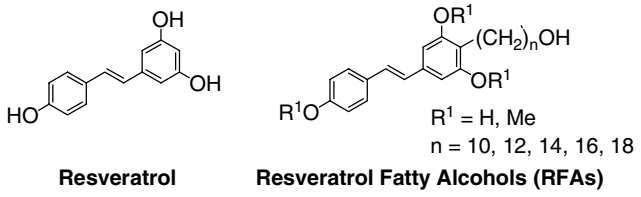


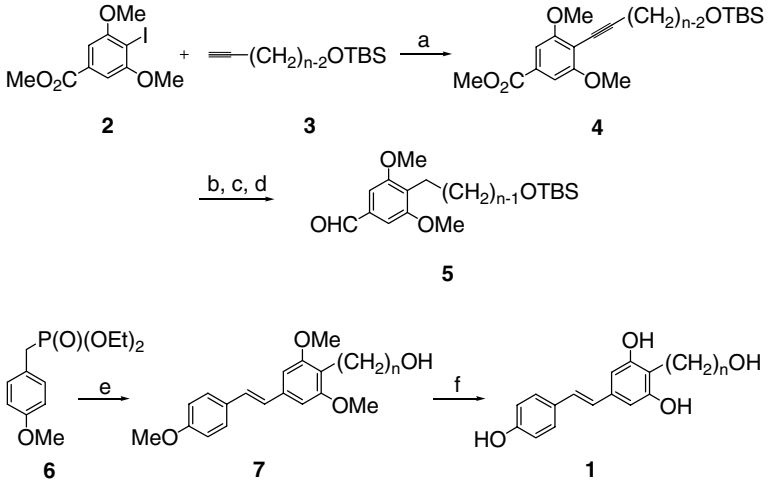
Figure 1. Structures of resveratrol and RFAs.

The synthesis of RFAs was based on two key steps. On one hand, a Sonogashira cross-coupling[16] between methyl 4-iodo-3,5-dimethoxybenzoate (**2**) and differently protected ω-alkynols **3** allowed us to couple the ω-alkanol side chains. On the other hand, a Wadsworth–Emmons[17] reaction between 3,5-dimethoxybenzaldehyde derivatives **5** bearing various ω-alkanol side chains and methoxybenzylphosphonate **6** gave methylated RFAs **7** which, after deprotection, yielded RFAs **1** (Scheme 1).

Our procedure started with commercially available resorcylic acid which was iodinated in presence of *N*-iodosuccinimide and then methylated with potassium carbonate and dimethylsulfate in refluxing acetone to give methyl 4-iodo-3,5-dimethoxybenzoate (**2**). The differently protected ω-alkynols **3** were prepared from *O*-protected bromo alcohols obtained by mono-bromination of the corresponding diols. Finally, the *O*-protected bromo alcohols in the presence of a lithium acetylide-ethylenediamine complex gave the desired ω-alkynols **3** in good overall yields (53–76%).

The Sonogashira cross-coupling between the protected ω-alkynols **3** and the methyl 4-iodo-3,5-dimethoxybenzoate (**2**) was realized in classical conditions and allowed us to obtain the different coupling products in good to excellent yields (Scheme 1). Then, catalytic hydrogenation with palladium on charcoal 5% followed by reduction with lithium and aluminium hydride yielded the corresponding hydrogenated benzyl alcohols. A Ley oxidation[18] gave then the desired benzaldehydes **5**, needed for the Wadsworth–Emmons coupling.

The final key step in the synthesis was the Wadsworth–Emmons reaction[17] between the different benzaldehydes **5** and the phosphonate **6** in the presence of sodium methylate which afforded the different series of methylated RFAs **7**. RFAs **1** were obtained after deprotection using boron tribromide. Phosphonate **6** was obtained in 80% in



Scheme 1. Synthesis of RFA analogues. Reagents and conditions: (a) Alkynols **3**, $PdCl_2(PPh_3)_2$, CuI, Et_3N, 80 °C, 24–48 h, 65–86%; (b) H_2, Pd/C 5%, EtOH, rt, 24 h, 90–98%; (c) $LiAlH_4$, THF, 0 °C to rt, 2 h, 92–99%; (d) TPAP, NMO, CH_2Cl_2, 0 °C to rt, 1 h, 81–94%; (e) i—NaOMe, DMF, 0 °C, 1 h; ii—**5**, DMF, 0 °C to rt, 1 h; iii—100 °C, 1 h; iv—HCl 2 N, rt, 17 h, 60–82%; (f) BBr_3, CH_2Cl_2, −78 °C to rt, 2 h, 46–76%.

a single step from the corresponding benzyl alcohols in presence of iodide in refluxing triethylphosphite.[19]

In order to evaluate the potential neuroregenerative activity of RFAs, their ability to influence the cell fate decision and the differentiation of neural stem cell derived neurospheres into neurons or glial cells was investigated. Neurospheres are undifferentiated cell aggregates floating in the culture medium which contain 5–10% neural stem cells (multipotent progenitors).[20] They arise from the cultivated NSCs due to selective action of growth factors like EGF and bFGF (brain FGF).[20]

After dissociation, neurospheres were grown up for three days (proliferation phase) and allowed to differentiate on a polyornithine support (differentiation phase) for a subsequent three days. RFAs were added at $T = 0$ (Fig. 2).[21] The differentiation was then analyzed by immunocytochemistry, using specific antibodies for, respectively, mitotic neurons [microtubule associated protein 2, MAP(2a+2b)], postmitotic neurons (monoclonal antibody against neuronal class III tubulin, TuJ1) and an astrocyte-specific intermediate filament protein (glial fibrillary acidic protein, GFAP). This study does not take into account oligodendrocytes. The total number of cells was quantified using TO-PRO-3-iodide, a nuclear marker (Fig. 3).

First of all, we determined that quantitatively the strongest effect on the neuronal population was observed at concentrations of 0.1 μM or 1 μM for most of the compounds. Higher concentrations (5 μM, 10 μM) were toxic and lower ones (1 nM) inefficient.

The efficiency to induce the differentiation of neurospheres into mature neurons of the most promising candidate RFA12 **1b** ($R^1 = H$, $n = 12$) was estimated quantitatively (Table 1). The qualitative aspect was estimated by the morphology of the neurons, including their size and the length of their extensions (Fig. 3).

Immunostaining showed that the length of the side chain and the presence of the hydroxyl group on the aromatic ring system were essential for a better biological activity. In fact, the most active compounds **7b** and **1b** are those bearing a ω-alkanol chain having 12 carbon atoms. Moreover, compound **1b**, RFA12 ($R^1 = H$, $n = 12$), is able to increase by 160% the number of neurons from the neurospheres compared to untreated cells

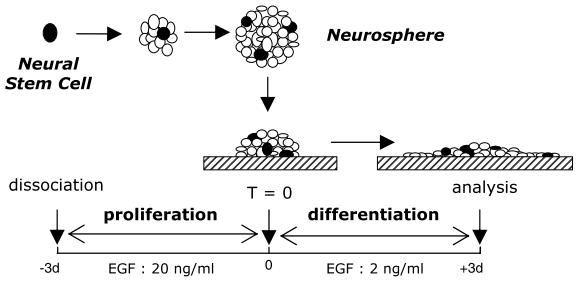


Figure 2. Experimental protocol for the neurosphere assay.

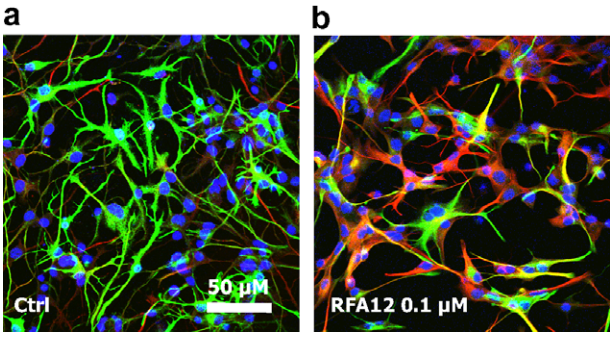


Figure 3. RFA12 induced differentiation of neural stem cell derived neurospheres into neurons. Neurospheres were allowed to differentiate for 3 days without (a) or with (b) RFA12 (0.1 μM), and immunostained with anti-MAP(2a+2b) and Tuj1 coupled to Alexa 555 for neurons (red), anti-GFAP coupled to Alexa 488 for astrocytes (green) and TO-PRO, a nuclei specific marker (blue).

(Table 1). Resveratrol alone does not enhance the neuronal production. RFA12 at 0.1 μM was more active than retinoic acid (RA) at 5 μM, known for its strong neurogenic capacity.[22] Finally, we confirmed that the ω-hydroxyl function was necessary for the activity as far as compound **8** has no effect on the number of neurons. These results comfort our starting hypothesis that the presence of a precise side chain length and the ω-alkanol function are necessary for the neurotrophic activity.

Besides, RFA12 increased in a concentration-dependent manner the number of neurons accompanied by a decrease of the number of astrocytes. In addition, in the

Table 1. Neuronal differentiation activity of RFAs[a]

Compound (0.1 μM)	R^1	n	% neurons versus control	% neurons versus RA[b]	Diff[c]
Ctrl			100	0.50	+
RA 5 μM			203	1	++
Resveratrol			105	0.52	+
7a	Me	10	147	0.72	+
7b	Me	12	188	0.93	++
7c	Me	14	135	0.67	+
7d	Me	16	107	0.53	+
1a	H	10	108	0.53	+
1b	H	12	263	1.30	++
1c	H	14	160	0.79	+
8	H	12[d]	106	0.52	+

+ indicates that neurospheres differentiate into neurons with a morphology similar to control conditions. ++ neurons are more differentiated, they elicit larger morphologies with longer process than in the control.

[a] Quantitative effect of RFAs, resveratrol and retinoic acid (RA) on neurosphere differentiation into neurons. Control (Ctrl) is EtOH. Each compound was evaluated in at least three independent experiments; variation was generally 20%.

[b] % of neurons divided by % of neurons when cells were treated with RA 5 μM.

[c] Diff stands for qualitative result on the neuronal differentiation of neurospheres.

[d] RFA without ω-OH.

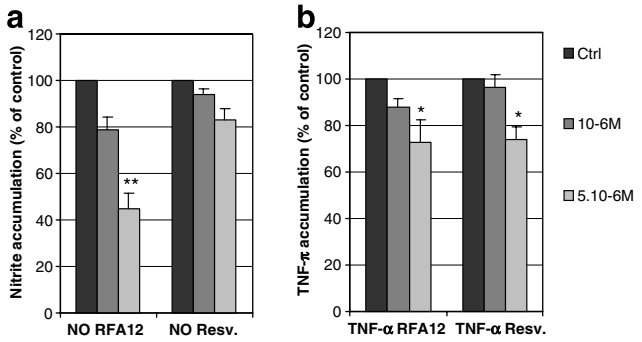


Figure 4. Effect of RFA12 and resveratrol (resv) on NO and TNF-α expression in MMGT-12 cells in presence of LPS (0.01 μg/mL). Culture supernatants were collected after, respectively, 48 h or 24 h incubation. NO and TNF-α production data are means ± SEM (bars) values obtained from three independent experiments ($n = 3$). Results are expressed as percentage of control (0.01 μg/mL LPS; (a) 100% = 5.57 nmol/mL NO and (b) 100% = 3731 pg/mL TNF-α. $^{*}p < 0.05$, $^{**}p < 0.01$, NO and TNF-α expression levels are significantly different from the corresponding control levels.

presence of RFA12, the neurons were larger and processes were longer which means that the neuronal precursors are more differentiated (Fig. 3). The fact that the increase in the number of neurons takes place at the expense of astrocytes suggests that RFA12 could specifically act on the cell fate.

The anti-inflammatory activity of our compounds was determined using a mouse microglial cell line (MMGT-12 type; a generous gift of Dr. E. Vanmechelen, Gent).[23] NO and TNF-α production, two common parameters related to inflammatory processes, were tested in parallel in this study.

MMGT-12 cells were activated by lipopolysaccharide (LPS) for 24 h or 48 h in the presence or absence of different concentrations of RFAs **1**, methylated RFAs **7** and resveratrol.

The accumulation of NO after 48 h was determined by the Griess method. The secretion of TNF-α, after 24 h of treatment, was determined using an ELISA.

Treatment of MMGT-12 cells in presence of methylated RFAs **7** did not show a significant decrease of NO or TNF-α production (results not shown). However, treatment with RFAs **1**, in particularly RFA12 **1b** ($R^1 = H$, $n = 12$), decreased NO (50%) and TNF-α production (30%) in MMGT-12 cells activated by LPS (5 μM) (Fig. 4). These results indicate that RFA12 **1b** has identical or even stronger anti-inflammatory activity than resveratrol. Thus, the presence of a specific side chain length is necessary for the biological activity.

In conclusion, we developed new compounds, Resveratrol Fatty Alcohols (RFAs), and particularly, RFA12, which cumulates neuroregenerative and neuroprotective activities. RFA12 can induce neuronal differentiation of neural stem cells while modulating microglial activation. RFA12 is a more efficient inducer for neuronal differentiation than RA or other tested synthetic compounds. Specifically, RFA12 can increase the number of neurons by 160%. Hence, we succeed in combining two biological properties in one molecule. A possible explanation of this effect is that our compounds act on the cell fate of the progenitors as the increase of neurons is accompanied by a decrease of astrocytes. This supposition suggests that RFA12 would act directly or indirectly on the Notch pathway that mediates a large array of cellular processes including the maintenance of stem cell self-renewal, proliferation and differentiation.[24,25] More systematic work is needed to completely elucidate the mechanism of action. Overall, this study indicates that this compound has a better pharmacological profile than resveratrol for the treatment of neurological diseases with degenerative and/or inflammatory components.

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