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Anticancer Activity of Vitamin E-Derived Compounds in Murine C6 Glioma Cells

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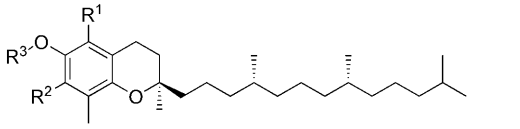
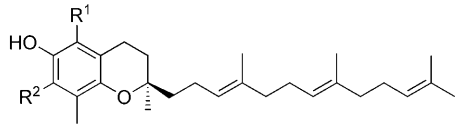
Vitamin E (VE)[1] is a family of eight structurally related compounds (four tocopherols and four tocotrienols) derived from chroman-6-ol. Originally discovered as a dietary factor essential



R^1 = Me,	R^2 = Me,	R^3 = H	α-tocopherol, **1**
R^1 = Me,	R^2 = Me,	R^3 = $COCH_2CH_2CO_2H$	αTOS, **2**
R^1 = Me,	R^2 = Me,	R^3 = CH_2CO_2H	αTEA, **3**
R^1 = Me,	R^2 = H,	R^3 = H	β-tocopherol, **4**
R^1 = H,	R^2 = CH_3,	R^3 = H	γ-tocopherol, **5**
R^1 = H,	R^2 = H,	R^3 = H	δ-tocopherol, **6**



R^1 = H, Me R^2 = H, Me α- to δ-tocotrienol

for reproduction in rats,[2] VE has many more important biological roles,[3] such as the scavenging of reactive oxygen and nitrogen species, thus protecting the organism against the attack of free radicals,[4] and the modulation of cellular signaling,[5] enzymatic activity and gene expression[6] in antioxidant and non-antioxidant manners.[7]

In recent years, evidence has accumulated on the antitumor and anti-inflammatory activity of VE metabolites[8] and synthetic derivatives.[9] The succinic monoester of α-tocopherol (**1**), α-tocopheryl succinate (αTOS, **2**), a representative VE analogue, has been reported as a potent cytostatic and cytotoxic agent in several cancer cell models, showing high selectivity for malignant cells and low toxicity to normal cells.[10,11] It was proposed that the chroman ring of **2** is responsible for the activation of specific signaling pathways, such as PP2A/PKC, leading to cell-cycle arrest or apoptosis, while the presence of the succinyl ester further increases these biological responses leading to cell and mitochondria membrane destabilization.[9d] Furthermore, methylation of the terminal free carboxylic group leads to loss of proapoptotic activity.[12] The limiting factor for the clinical application of **2**, in particular regarding oral administration, is that the ester bond is prone to cleavage by esterases, releasing α-tocopherol, which is not active as an anticancer agent. In that regard, α-tocopheryloxyacetic acid (**3**, αTEA) is a promising VE analogue, which, unlike **2**, has an acetic acid moiety attached to the chroman hydroxy group via a non-hydrolyzable ether bond. Compound **3** was shown to be very effective in terms of selectivity and potency in suppressing the growth of various tumors, both in vitro and in vivo.[13,14]

Recently, other VE analogues with non-hydrolyzable bonds were reported with enhanced proapoptotic activity against Jurkat, U937 and human Meso-2 malignant mesothelioma cells compared to compound **2**.[15,16] While structurally related to **2**, the ester bond is replaced by an amide bond, the precursors being α- and δ-tocopheramine. Apart from the expected increased stability, a substantial improvement in the apoptotic activity is observed when going from the ester to the amide analogues and introducing an olefinic bond in the acid moiety. Those results suggest that the amide modification can be used as a starting point for the design of new VE analogues with potent antitumor activity.

The molecular mechanism that causes the induction of apoptosis by VE analogues is still unclear. Monosuccinate **2** leads to destabilization of lysosomes and mitochondria by sphingomyelinase activation, ultimately targeting mitochondria through the generation of reactive oxygen species (ROS),[17] affording the release of cytochrome c and the activation of proapoptotic proteins, such as caspase 9 and the Bcl-2 protein family.[9d] Apoptosis induced by the ether analogue **3** seems to proceed through another mechanism involving death receptor Fas signaling, the activation of JNK and its substrate c-Jun,[18] truncation of Bid, conformational change of Bax, and activation of caspases 9 and 3.[19]

Our research involves the systematic study of the application of VE and related compounds in the treatment of glioblastoma—one of the most frequently occurring brain tumors, accounting for ~12–15% of all brain tumors.[20] Glioblastomas are among the most aggressive malignant human tumors, characterized by a diffuse local invasion of the normal parenchyma that renders complete surgical resection of cancerous tissue

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extremely difficult. Furthermore, the currently available drugs are of poor therapeutic potential.[21] Thus, novel therapeutic strategies are required to improve the poor prognosis associated with this kind of tumor.

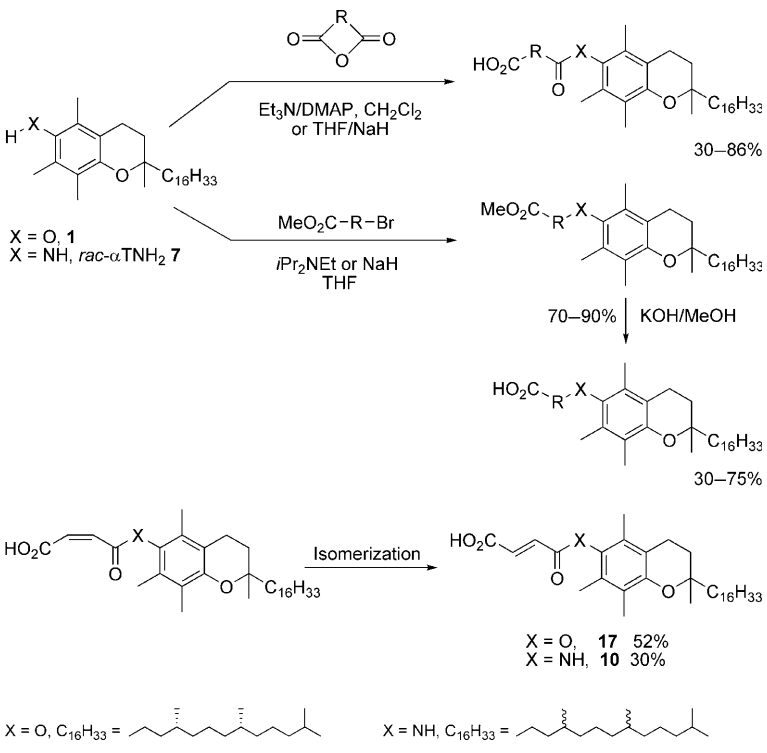
In previous studies, we demonstrated that 13′-carboxy-δ-tocotrienol, γ-tocopherol, α- and γ-(2′-carboxyethyl)-6-hydroxychromans (metabolites of α- and γ-tocopherol, respectively) act as antiproliferative agents on murine glioma C6 cells.[9d,22] This biological activity is a consequence of cell-cycle arrest in G0/G1 phase, which is mediated by decreased expression of cyclin E and cyclin-dependent kinases 2 and 4, and the phosphorylative activation of p27 (specific inhibitor of cells entering S phase). Thus, considering the perspective of a possible chemotherapeutic application of VE in human gliomas and the promising results provided by the VE amides discussed above, we wanted to investigate the activity of the VE amides and other analogues on a glioma C6 cell model. Therefore, we prepared a small library of VE derivatives with a free acid group linked to the chroman core via an amide, ether or ester bond, and assayed them for antiproliferative and apoptotic activity.

By applying the general synthetic strategy shown in Scheme 1, VE analogues were obtained by reacting α-tocopheramine **7**[23] or the α-tocopheryloxyl anion with the appropriate cyclic anhydride or bromoester (see Table 1 for structures). In the latter case, saponification of the ester afforded the acid derivative. Monoester **16** was obtained in improved yields by generating the α-tocopheryloxyl anion in situ using NaH rather than using Et_3N and DMAP under classic conditions, whereas the latter procedure conveniently provided amides **8**, **9**, **11** and **12**. Amino acid **14** was synthesized by a similar approach by reacting **7** with methyl (*E*)-4-bromo-2-butenoate in the presence of *N*,*N*-diisopropylethylamine, but the subsequent alkaline hydrolysis with methanolic KOH provided the acid **14** in low yield together with compound **15** as the major product. However, this apparent drawback turned out to give us another VE analogue for the screening test. The ether analogues **18**, **20**, **3** and **21** were prepared by reacting **1** with NaH, followed by the addition of methyl (*E*)-4-bromo-2-butenoate, methyl 4-(bromomethyl)benzoate and ethyl bromoacetate, respectively. The subsequent alkaline hydrolysis of the ester group afforded the acid analogues. Fumaric monoester **17** was obtained by isomerization of **16** by refluxing in CCl_4 in the presence of a catalytic amount of benzyltrimethylammonium tribromide. A stoichiometric amount of the latter reagent was used in the bromination of **9**, affording amide **13**. Derivative **10** was obtained by acid-catalyzed isomerization of **9**. Monoester analogues of amides **11**, **12** and **13** proved to be unstable under purification conditions and could, therefore, not be isolated.

All synthesized VE analogues were evaluated in an MTT assay[24] for their effect on C6 cell viability. The data showed a marked reduction in cell viability for all VE analogues when tested at 1 μM, while the tocopherols (**1**, **4**–**6**) provided poor to low antiproliferative activity at the same concentration. Most of the VE analogues exhibited submicromolar IC_{50} values (Table 1), and in many cases the values were lower than that of temozolomide **22**, the most commonly used drug in the treatment of gliomas.

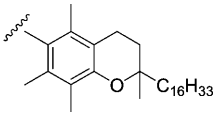
In contrast to the results reported using other tumor cell lines,[15] the substitution of the ester with the amide or amino bond was slightly detrimental to the cytotoxic activity of these compounds in C6 cells (**8** vs **2**, **14** vs **18**, **9** vs **16**). Moreover, the introduction of electron withdrawing groups in the tocopheramine derivatives (**11**–**13** and **15**) further decreased the antiproliferative activity of these compounds.

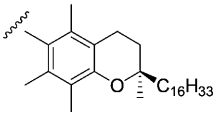
The presence of an olefinic double bond between the free acid group and the chroman ring in the ester and ether analogues did not improve activity compared with the very good potency exhibited by compounds **2**, **3** and **21**. Conversely, the results provided by compounds **19** and **20** were quite promising, as, to the best of our knowledge, they are the first examples of succinate-like VE analogues with an aromatic group introduced in the acid portion. In fact, the aromatic group can be further functionalized easily, allowing the development of other VE derivatives with potential antitumor activity.

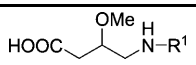
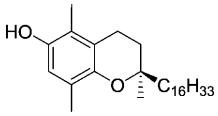
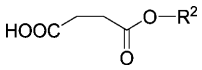
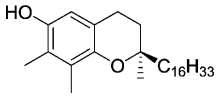
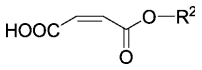
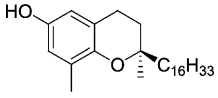
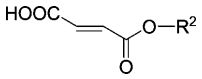
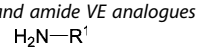
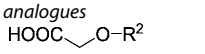
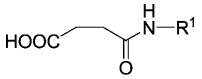
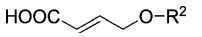
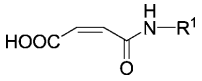
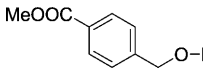
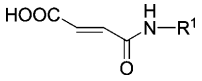
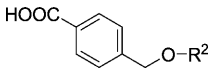
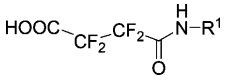
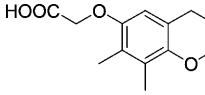
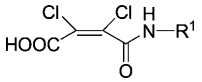
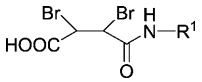
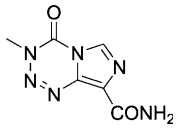
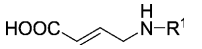


Scheme 1. General synthetic strategy for the preparation of VE analogues.

Table 1. Individual IC_{50} values for the effect of vitamin E compounds on C6 cell viability determined by the MTT test.[a]

R^1 = 

R^2 = 

Compd	VE analogue	$IC_{50} \pm SD$ [μM]	Compd	VE analogue	$IC_{50} \pm SD$ [μM]
tocopherols			**15**		1.28 ± 0.11
1		> 50	*ester VE analogues*		
4		> 50	**2**		0.11 ± 0.01
5		2.00 ± 0.13	**16**		0.21 ± 0.02
6		> 50	**17**		0.85 ± 0.07
amino and amide VE analogues			*ether VE analogues*		
7		0.41 ± 0.04	**3**		0.36 ± 0.04
8		0.53 ± 0.05	**18**		0.74 ± 0.06
9		1.04 ± 0.08	**19**		0.52 ± 0.04
10		0.68 ± 0.07	**20**		0.45 ± 0.04
11		1.57 ± 0.14	**21**		0.19 ± 0.02
12		0.91 ± 0.08	*temozolomide*		
13		0.86 ± 0.09	**22**		0.74 ± 0.08
14		1.25 ± 0.10			

[a] IC_{50} value indicates how much VE analogue is needed to reduce cell viability by 50% compared to a control. Data represent mean values of at least five separate experiments.

Moreover, although the presence of a free carboxylic group in VE analogues has been widely reported as a prerequisite for antitumor activity,[9c,12] this does not appear to be the case for compounds **19** and **20.** We were also quite surprised by the good activity afforded by tocopheramine **7**, a close structural analogue of **1**, which was shown to be approximately 100-fold more effective than the inactive compound (**1**).

We also investigated the number of necrotic or apoptotic cells after 24 h of incubation with 1 μM of test compound by cytofluorimetric analysis (Table 2). Tocopherols **1**, **4**–**6** affected the cell viability in a negligible way, while the other VE derivatives provided a moderate to high induction of apoptosis: 18–43% (VE amides) and 5–75% (VE esters and ethers). Among the VE amide analogues, the best apoptosis (A) to necrosis (N) ratio was exhibited by cytostatic compound **12** (A/N = 4.3), while for VE ester and ether analogues, a ratio of 13.5 was provided by compound **19**, followed by compound **16** (A/N = 6.4) and **3** (A/N = 5.0). These three compounds also showed the highest pro-apoptotic activity (**3**, 75%; **19**, 65%; **16**, 58%). Conversely, compound **2** caused the highest necrotic effect (85%) at the concentrations tested in this study. This result is in contrast to data reported in other cell lines.[10]

To investigate the effect of these compounds on the cell cycle, further cytofluorimetric analysis was carried out (figure 1S, Supporting Information). This analysis revealed that compounds **9** and **11**–**15** essentially caused a cytostatic effect (cell count: 40–60%, Table 2), compounds **7**, **8**, **10**, **17** and **18** showed moderate antiproliferative activity (cell count: 30–40%, Table 2), while compounds **2**, **3**, **16**, **19**, **20** and **21** proved to be very effective inhibitors of cell growth (cell count: < 30%, Table 2), succinate **2** showed the highest value.

In conclusion, a series of VE analogues, half of them never described before, were assessed for their anticancer activity in murine C6 glioma cells. The substitution of the ester or ether bond with an amide bond to link the free acid group to the chroman core of VE analogues did not increase their cytotoxicity in this cell model. However, nine compounds exhibited IC_{50} values better than temozolomide, a golden standard in the treatment of glioblastoma. While compound **2** provided the lowest IC_{50} value, this was due mostly to its necrotic activity. However, compound **3** showed a very good IC_{50} value and the highest pro-apoptotic effect; similar properties were found for compounds **16** and **21**. Contrary to what has generally been reported for VE analogues,[9c] the presence of a free acid group did not appear to be an essential requirement for antitumor activity in C6 cells, as shown by amine **7** and ester **19**. The latter showed good growth inhibition of the cancer cells and the highest apoptosis to necrosis ratio, representing a

Table 2. Effects of compounds **1**–**22** on cell death parameters of murine C6 glioma cells.[a]

Compd	Cell count[b] [%]	Residual cells[c] [% of total] V	A	N	A/N
CTRL	100±2	94±1	4±2	2±1	2
tocopherols					
1	92±3	88±2	8±2	4±2	2.0
4	91±3	84±3	9±2	7±3	1.3
5	67±4	89±1	7±1	4±2	1.8
6	71±2	72±3	16±2	12±3	1.3
amino and amide VE analogues					
7	36±3	46±5	39±6	15±5	2.6
8	32±2	57±4	33±4	10±4	3.3
9	42±3	33±4	32±4	35±4	0.9
10	38±3	48±6	35±6	17±5	2.1
11	48±2	50±5	33±6	17±6	2.8
12	44±4	47±7	43±8	10±7	4.3
13	49±3	73±3	18±3	9±3	2.0
14	44±4	52±4	31±5	17±5	1.8
15	52±3	57±4	28±4	15±4	1.9
ester VE analogues					
2	14±3	10±4	5±4	85±3	0.1
16	23±3	33±6	58±5	9±5	6.4
17	37±2	40±4	40±4	20±4	2.0
ether VE analogues					
3	26±3	10±5	75±4	15±5	5.0
18	39±2	42±4	37±4	21±4	1.8
19	21±2	30±5	65±5	5±5	13.0
20	25±3	40±5	42±4	18±5	2.3
21	21±3	29±4	50±5	21±5	2.4
temozolomide					
22	23±3	45±4	31±5	24±5	1.3

[a] Cell count, viability, apoptosis and necrosis were evaluated by the cytofluorimetric assay after 24 h of exposure to each test compound at the final concentration of 1 μM. Data represent mean values of at least three separate experiments. Cell count% = percentage cell count with respect to cell count of control-vehicle. V = viable, A = apoptotic, N = necrotic, % RSD = percent relative standard deviation, CTRL = vehicle control. [b] Cell count is given as percent of CTRL ±% RSD. The number of cells in the vehicle control doubled after 24 h, thus a cell count of 50% means that there was no change in the number of cells after 24 h, thus a cytostatic effect. [c] Values given are all ±% RSD.

slight improvement over the corresponding acid **20**. These results are very promising for the design of new VE analogues for the treatment of glioma, considering the various possibilities of functionalization of the aromatic moiety linked to the chroman ring in analogues **19** and **20**.

Quite unexpectedly, tocopheramine **7**, which is not a succinate-like or ether-linked VE analogue, showed good antiproliferative and apoptotic properties. Notably, just substituting the hydroxy group in the inactive derivative **1** with an amine group led to a 100-fold increase in antitumor activity. Further studies are currently underway to investigate the mechanism-of-action of these VE analogues and their specific targets, and also the therapeutic potential of the other tocopheramines and tocotrienamines that have been recently prepared in our laboratories.[23]

Acknowledgements

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Keywords: amides · antitumor agents · apoptosis · cancer · vitamin E

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