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Coumarin derivatives protection against ROS production in cellular models of Aβ toxicities

CHRISTOS A. KONTOGIORGIS[1,2], YANAN XU[2], DIMITRA HADJIPAVLOU-LITINA[1], & YUAN LUO[2]

[1]*Aristotle University of Thessaloniki, School of Pharmacy, Department of Pharmaceutical Chemistry, 54124, Thessaloniki, Greece, and* [2]*University of Maryland, Department of Pharmaceutical Sciences, Baltimore, MD21201-1180, USA*

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Abstract
The role of oxidative stress and free radicals in the development of Alzheimer's disease (AD) has been the focus of many recent studies. The role of hydrogen peroxide (H_2O_2) in AD is thought to be associated with Aβ (amyloid – β) damage in cells. A number of coumarin derivatives were previously found to be potent anti-inflammatory and antioxidant agents. Herein, these coumarin derivatives were tested as H_2O_2 scavengers with the DCF assay using two types of neuronal cells: (a) wild type (N2a) neuroblastoma cells and (b) APP/PS1 transgenic cell line expressing Aβ. Their scavenging activity was varied between the types of cell cultures and it was found to be concentration and time dependent in the mutant cells. Their protective role against cell death further supports this notion. These results suggest that these compounds could be used as a template in the design of new molecules with a possible role in AD.

Keywords: *Coumarin, hydrogen peroxide scavengers,* β*-amyloid peptide, N2a neuroblastoma cells, Δ.9 mutant cells*

Introduction

Cumulative oxidative stress and the role of free radicals in the development of Alzheimer's disease (AD) and impairment of brain function has been the focus of many recent studies. Ageing has interestingly been postulated to be the greatest risk factor associated with the development of AD [1,2]. Research indicates that cellular insults resulting from free radicals may be a major contributor to the neurotoxicity and pathology of AD [3]. With recent findings suggesting links between AD, deposition of Aβ [4,5] and oxidative stress [6], much attention has been devoted currently to antioxidant research.

Alzheimer's disease is characterized by the formation of senile plaques [7]. Amyloid β-peptide (Aβ), the major constituent of the senile plaques (SP) [8], plays an important role in the pathogenesis of AD [9]. Aβ is a 39–43 amino acid peptide derived from proteolytic processing of amyloid precursor protein (APP), a transmembrane glycoprotein that is expressed during normal cellular metabolism [10]. Synthetic Aβ is toxic to neuronal cultures [11] and its neurotoxic properties may be involved in the neuronal degeneration seen in AD [12]. Experimental and genetic results point to an essential role of Aβ in AD [13]. Mutations in the APP encoding gene are found in early-onset AD patients [13] and they are associated with excess Aβ deposition and oxidative stress [14]. Transgenic animals over-expressing mutant APP show increased Aβ content in the brain and exhibit oxidative stress [15].

Correspondence: Dr Christos Kontogiorgis, Aristotle University of Thessaloniki, School of Pharmacy, Department of Pharmaceutical Chemistry, 54124, Thessaloniki, Greece. Tel: +302310997627. Fax: +302310997612. E-mail: kontog@pharm.auth.gr

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Reactive oxygen species (ROS) have been shown as causative factors involved in many human degenerative diseases and antioxidants have been found to have some degree of preventive and therapeutic effects on these disorders [16,17]. Reactive oxygen radicals are by-products of various endogenous processes that can be stimulated by external factors, such as irradiation and other agents such as xenobiotics and polyphenols [18]. The free radical generation may result directly from inhibition of the mitochondrial respiratory chain or indirectly during the apoptotic process itself and leads to the production of superoxide radicals that generate other ROS, such as hydrogen peroxide (H_2O_2) or hydroxyl radicals; they damage mitochondria and can leak into the cytosol to damage other organelles [19].

Hydrogen peroxide (H_2O_2), one of the main ROS, causes lipid peroxidation and DNA oxidative damage in cells [16,20]. Controlled generation of hydrogen peroxide appears to be a common phenomenon among different cell types and may play an important role in signalling pathways. Since H_2O_2 is able to pass freely across cell membranes, it can transfer information to nearby cells or tissues acting in a paracrine fashion, similar to nitric oxide [18]. A large number of molecules have been proposed or identified as direct or indirect targets of intracellular H_2O_2.

Evidence for involvement of free radicals in AD include the presence of elevated levels of protein oxidation, lipid peroxidation products and oxidative damage to mitochondria in AD brain [17]. The ability of toxic Aβs to induce protein oxidation and inhibit the activity of oxidation-sensitive enzymes is consistent with the hypothesis that Aβ can act like a prooxidant [17]. In addition to numerous reports supporting the free radical hypothesis in Aβ toxicity, a sizable body of evidence supports the involvement of free radicals indirectly. Furthermore, oxidative stress appears to mediate Aβ toxicity by free radical production [21]. Brain neurons are particularly vulnerable to H_2O_2 because of their relatively low levels of antioxidant enzymes and dependence on mitochondrial respiration [16].

Antioxidants protect against the radicals and it is important to balance an enhanced radical production with a sufficient supply of antioxidants. [18]. Antioxidant molecules such as N-acetyl_L-cystein (NAC), which was found to protect neuroblastoma cells from oxidative stress (induced by hydrogen peroxide and other stressing parameters) [21], vitamin E and catalase appear to protect cells from oxidative insults and the resultant induction of apoptotic cell death [21]. As a biological antioxidant, vitamin E prevents the oxidation of polyunsaturated fatty acids and proteins and is an important protective factor in the development of diseases and morbidity related to oxidative processes [22]. Unfortunately, since lipid-soluble vitamins are not easily excreted, excessive intake of vitamin E has been associated with potentially harmful side effects [22].

Alzheimer's disease as neuroinflammatory disease

Alzheimer's disease tissue is in general characterized by neuroinflammatory changes, which are observed in both sporadic and familiar AD as well as in some other forms of dementia in other neurodegenerative diseases. The chronic inflammation is not causative, although it may greatly influence the pathogenesis, in which case there may be potential for anti-inflammatory therapy. Increasing experimental evidence supports the view that inflammation-generated oxidative stress contributes to the lesions of AD [23].

Just as has been observed with oxidative stress, increased sensitivity to inflammation is also seen in ageing. Studies indicated that this inflammatory marker (5-LOX) is expressed in CNS neurons and may be involved in neurodegenerative processes. Findings indicate that 5-LOX may exert its actions through tyrosine kinase receptors and cytoskeletal proteins [24] and it is known that 5-LOX gene expression and activity are increased in ageing. Cyclooxygenase and 5-lipoxygenase inhibitors can protect the neurotoxicity [25], presumably by reducing 5-LOX expression.

Additionally, studies indicate that the expression of one form of COX-2 appears to be associated with amyloid beta deposition in the hippocampus [26,27]. Since the PG synthesis pathway appears to be a major source of reactive oxygen species (ROS) in the brain [28] and in other organ systems, these findings indicate that inflammation may be accompanied by and even generate its 'evil twin' or in producing the deleterious effects of ageing. Thus, such factors as cytokines, cycloogenases, prostaglandins, etc. may act as extracellular signals in generating additional ROS that are associated with decrements in neuronal function or glial neuronal interactions and ultimately the deficits in behaviour that have been observed in ageing [29].

Recent advances in AD treatment involve the investigation of non-steroidal anti-inflammatory drugs (NSAIDs) in reducing prevalence of AD among users [30]. However, chronic use of these preparations has been shown to cause side effects such as gastrointestinal toxicity [31]. Given the occurrence of side effects with long-term use, increased interest has turned towards the use of alternative remedies.

Epidemiological evidence shows that NSAIDs could be effective if used at an early stage of the disease. However, for this possibility to be realized, appropriate agents must be selected. It has been

presented that selective COX-2 inhibitors are an inappropriate choice, due to the evidence of exacerbation of neuronal death in some animal neurotoxicity models following COX-2 inhibition [32]. On the other hand, clinical trials of COX-1 inhibitors show that they could be much more active [23]. It is also proposed that a new age of COX-1 inhibitors NSAIDS, based on introducing *a nitrate ester* moiety, called NO-NSAIDs, may circumvent the side effect problems [33].

Coumarin derivatives and Alzheimer's disease

Coumarins comprised a group of phenolic compounds widely distributed in natural plants and they have recently attracted much attention because of their pharmacological activities. Coumarins have been reported to have multiple biological activities [34]. They have been used to treat such diverse ailments as cancer, burns, brucellosis, cardiovascular and rheumatic diseases [35]. The coumarin molecule has been shown to possess unique antioedema and anti-inflammatory activities. Various coumarin related derivatives are not only recognized as inhibitors of the lipoxygenase and cyclooxygenase pathways of arachidonate metabolism [36–38], but also of neutrophile dependent superoxide anion generation [39]. Several natural or synthetic coumarins with various hydroxyl and other substituents were found to inhibit lipid peroxidation and to scavenge hydroxyl radicals and superoxide anions [40] and to influence processes involving free radical-mediated injury, as can some plant phenolics and flavonoids [41].

A series of natural products and extracts containing coumarin derivatives have been found to be active. It has already been reported that ethanolic extract of A. gigas Nakai (Umbelliferae) (included coumarins) and decursinol, a coumarin isolated from A. gigas, shows a high inhibitory activity toward acetylcholinesterase [42] and they both were found to prevent against $A\beta_{1-42}$ induced memory impairment in mice [43].

Many coumarins, such as fraxetin, showed scavenging activity against reactive oxygen species and inhibited lipid peroxidation in rat brain [44]. Although the physiological benefits of flavonoids and other natural products have been largely attributed to their antioxidant properties in plasma, coumarins may also protect cells from various insults. It has been shown that fraxetin can protect neuroblastoma cells from the damage induced by reactive oxygen species. The neuroprotective effects of fraxetin may be partly associated with its antioxidant properties [17].

Esculetin (6,7-dihydroxycoumarin) showed hydroxyl radicals scavenging activity and inhibited lipid peroxidation in rat livers [45]. In previous studies we have presented a series of coumarin–Mannich bases and a series of coumarin derivatives with an azomethine linkage at position 7 of the coumarin system (Table I[46–48]. All these coumarin derivatives have been tested for their anti-inflammatory *in vivo* activity and for their antioxidant *in vitro* activity. Some of them were found to highly interact with DPPH and scavenge hydroxyl free radicals and superoxide anion radical.

In this follow-up study of our previous studies, we sought to characterize anti-oxidative properties of new coumarin derivatives in a cellular model associated with Alzheimer's disease in comparison with other known antioxidants. The mutant rat neuroblastoma cells line APPswe/PSd9 N2a conditionally expresses Aβ [49]. Studies of Aβ toxicity using the cell culture models have contributed tremendously to the understanding of AD pathogenesis processes [50,51] and effects of neuroprotectants [52]. Compared with other available models of AD, the mutant N2a cells allow easy propagation and pharmacological manipulation [53]. In the present study, the compounds were tested in (a) wild type neuroblastoma cells (N2a), (b) wild type cells induced with hydrogen peroxide (H_2O_2) in different concentrations, (c) mutant cells Δ9 without butyric acid induction for transgene expression and (d) mutant cells Δ9 induced with butyric acid. The cells viability was studied when hydrogen peroxide was induced. A better understanding of the antioxidative properties of these coumarin derivatives may provide crucial information for further design and synthesis of new analogues with potent role in neurodegenerative diseases as well as those associated with ageing processes.

Materials and methods

Reagents

Coumarin derivatives 2–12 and 14–20 were previously synthesized [46–48]. Compound 1 was 7-hydroxy-coumarin (umbelliferone) and compound 13, 7-methyl-coumarin. The parent coumarin molecule, compound 21, was used as a reference compound. Coumarin derivatives were dissolved in DMSO and, for the final concentrations, they were dissolved in culture media. The final concentration of DMSO was no more than 0.2%. L-ascorbic acid, Vitamin E and NAC (N-acetyl-L-cysteine) were purchased from Sigma (St. Louis, MO). The fluorescent probes 2′,7′-dichlorohydrofluorescein diacetate (DCFH-DA) was purchased from Sigma. MTT (mehtylthiazoletetrazolium) was purchased by Calbiochem (San Diego, CA).

Cell cultures

The wild type neuroblastoma control cells (N2a), and the N2a cell line stably expressing the double

mutations of Swedish mutation APP695 and the exon-9 deletion mutant PS1 (swe/Δ9) were used [54]. The cells were maintained as described previously by Thinakaran et al. [49], in medium containing 50% Dulbecco's Modified Eagle Medium (DMEM) and 50% Reduced Serum Modified Eagle Medium (Opti-MEM), supplemented with 5% foetal bovine serum (FBS) and 1% other antibiotics (Invitrogen, Grand Island, NY). In the case of Δ.9 mutant cells, G418 were added to the medium to give a final concentration of 200 μg/ml.

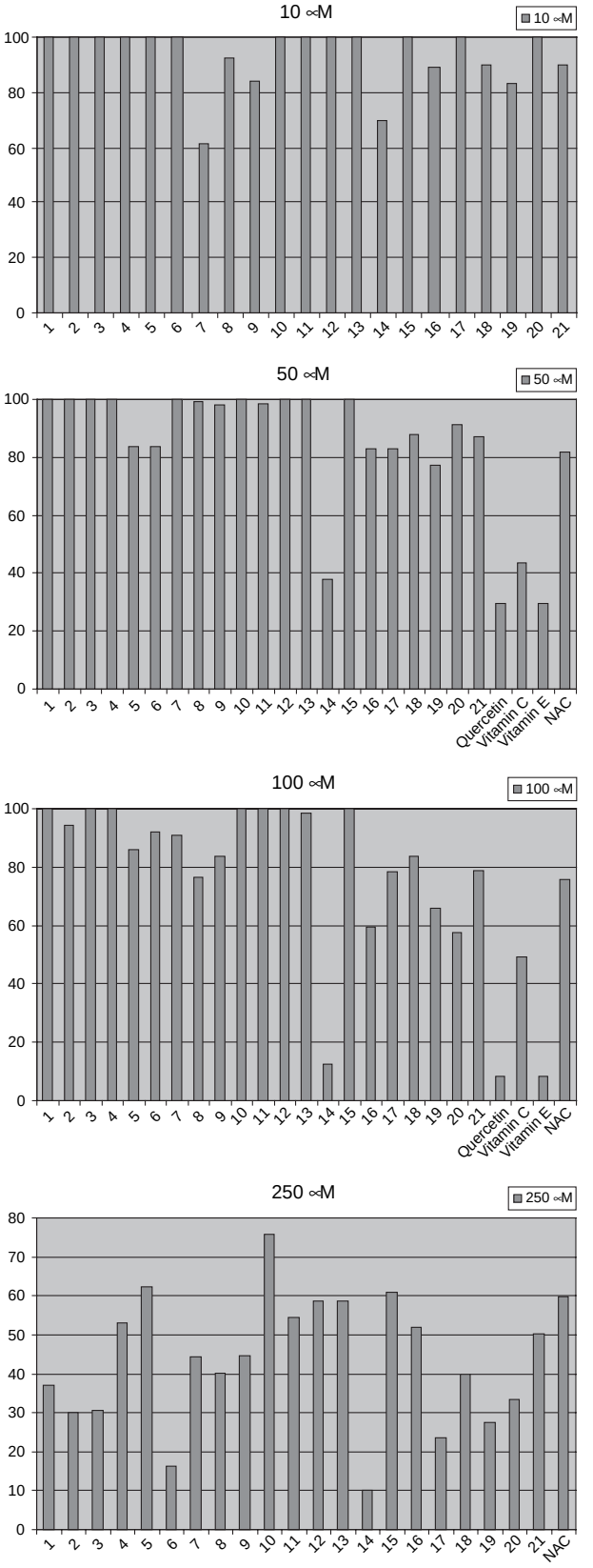


Figure 1. (*Continued*)

Analysis of oxidative free radicals

Intracellular ROS were measured in cultured cells using 2′,7′-dichlorodihydrofluroscein diacetate (H_2 DCFDA; Molecular Probes, Eugene, OR). Non-fluorescent DCF-DA is a freely cell permeable dye, which is readily converted to fluorescent 2′,7′-dihydrofluorescein (DCF) due to the interaction with intracellular peroxide (H_2O_2) [55]. The principle of this assay is that DCFH-DA diffuses through the cell membrane and is enzymatically hydrolysed by intracellular esterases to non-fluorescent dichlorofluorescein (DCFH) which reacts with H_2O_2 to form a fluorescent compound: dichlorofluorescin (DCF). The blue fluorescent dye HE is oxidized by O_2^- to ethidium, which stains the nucleus a bright fluorescent red. At the end of the specified treatment times, cell samples were incubated in the presence of 50 μm H_2DCFDA in phosphate buffered saline (PBS) at 37°C in an FL × 800 Microplate Fluorescent Reader (Bio-Tek Instruments, Winooski, VT) for quantification of fluorescence at excitation 485 nm and emission 530 nm. Cell samples were read every 15 min for 2 h.

There have been several attempts to directly detect the intracellular generation of hydrogen peroxide in UVA-irradiated cells by loading them with DCFH and following the appearance of DCF [56]. However, we have shown previously that DCF undergoes photoreduction upon visible irradiation to generate a semi-reduced DCF radical that reacts with oxygen to form superoxide and ultimately H_2O_2 [57,58].

Figure 1. ROS levels in wild type (N2a) neuroblastoma cells. Cells were assayed for ROS levels with results expressed as percentage of fluorescence (% DCF) relative to untreated controls (determined as 100%). Mutant cells have been treated with the coumarin derivatives in four different concentrations (10, 50, 100 and 250 μm) for 24 h. Standard cell culture conditions as described in the Methods section, are expressed as percentage of fluorescence relative to untreated control cells as 100%. Quercetin, a natural flavonoid product, was also used in different concentrations as a reference compound (5 μg/ml: 40%, 10 μg/ml:22% and 50 μg/ml: 13.3%). Vitamin C, Vitamin E and NAC have been used as reference compounds. * Results were obtained from at least 3 independent samples for each compound test.

Cell culture treatment

Cells were cultured in 96-well plates at about 2000 cells in 200 μl media per well. Cells were pre-treated with compounds (in different concentrations) in culture medium for 24 h.

Cell viability

Wild type neuroblastoma cells (N2a) were seeded in 96-well plates and when the cells reach 80% confluency, they were incubated for 2 h with 100 μm H_2O_2. The medium was then replaced with normal growth medium for 24 h as indicated in figure legends with cotreatment of coumarin derivatives in two different concentrations. The number of viable cells at each time point was determined with the mehtylthiazoletetrazolium (MTT) assay. The assay determines the number of viable cells by bioproduction of MTT into a coloured formazan product which is detected by absorbance at 570 nm with a 96-well plate reader. Cell viability as a percentage of control (cells incubated in normal medium only). MTT is a water soluble tetrazolium salt yielding a yellowish solution when prepared in media or salt solutions lacking phenol red. Dissolved MTT is converted to an insoluble purple formazan by cleavage of the tetrazolium ring by mitochondrial dehydrogenase enzymes of living cells [59]. This water insoluble formazan can be dissolved in dimethylsulphoxide (DMSO) and measured spectrophotometrically.

Results

Intracellular levels of H_2O_2 in wild type neuroblastoma cells treated with coumarin derivatives

To determine anti-oxidative activities of the *coumarin derivatives*, we first conducted DCF assay to detect levels of H_2O_2 in wild type cells treated with coumarin derivatives in four different concentrations (10, 50, 100 and 250 μm) It seems that all the coumarin derivatives presented moderate anti-oxidative activity at the lowest concentration (Figure 1). The most active compounds were found to be compounds 9 and 14, 16 and 20, which showed medium activity at the highest concentration (250 μm, range 30–60%). Vitamin C (L-ascorbic acid), Vitamin E, (a-tocopherol), Quercetin (as natural product) and NAC were used as controls of known antioxidants.

After identification of the most active compounds among coumarin derivatives tested, we then determined time course of compounds 8, 14 and 16 in wild type cells treated at a concentration of 100 μm for 3, 6, 12 and 24 h. The results are given in Figure 2. The compounds presented a decrease in activity after 3 h.

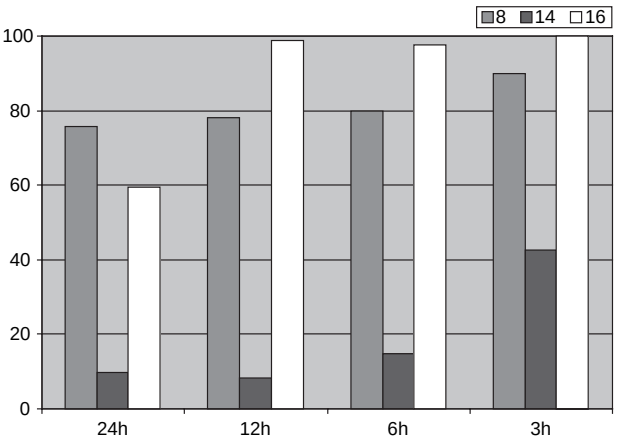


Figure 2. The neuronal cells were treated with coumarin derivatives 8, 14, 16 (100 μm) in culture medium over a range of time periods (3–24 h, as indicated) followed by measurement of ROS levels. Data are expressed as% DCF of ROS relative to untreated controls (as 100%). * Results were obtained from at least 3 independent samples for each compound test.

Effect of coumarin derivatives on intracellular levels of H_2O_2 in Δ.9 mutant cells without transgene expression

The intracellular levels of H_2O_2 in neuronal cells are associated with the expression of Alzheimer's disease. Thus, we tried to define if the coumarin derivatives can behave as antioxidants without the Aβ expression. Δ.9 mutant cells were treated with coumarin derivatives for 24 h and the intracellular ROS levels were determined with the DCF assay. The data presented in Figure 3 demonstrate that some coumarin derivatives showed significant antioxidant activity, with compounds 5, 11, 13, 18, 19 and 20 being the most active.

Effect of coumarin derivatives on intracellular levels of H_2O_2 in Δ.9 mutant cells with transgene expression by butyric acid

It has been suggested, according to the Aβ-induced oxidative stress theory of AD, that internal expression of Aβ would cause a rise in the levels of ROS [22]. One has previously shown significantly higher ROS levels in an AD-associated cellular model compared to wild type counterparts. Aβ-secreting mutant cells (swe/Δ9) showed ~3.13-fold higher ROS levels than N2a wild type cells (reference, Smith 2003?).

In this assay the cells were treated for 24 h with coumarin derivatives at four different concentrations (10, 50, 100 and 250 μm). The data are given in Figure 4. It is important to mention that many coumarin derivatives exhibited very high free radical scavenging activity. Compound 14 was found to be the most active compound under these experimental conditions. For most of the compounds, a higher concentration (250 μm) correlated with a more

significant scavenging activity. However, some coumarin derivatives (3, 15, 16 and 19) were found to be very active even in the concentration of 100 μm. Vitamin C, Vitamin E, Quercetin and NAC were used as positive controls.

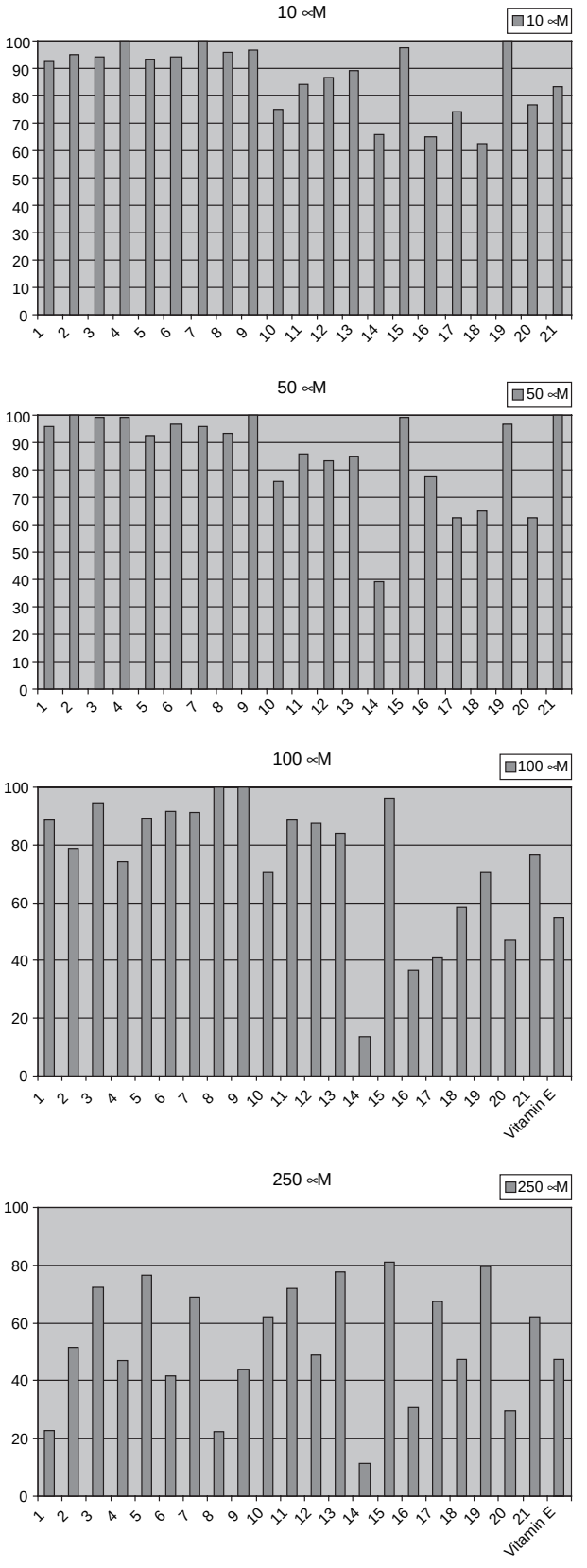


Figure 3. (*Continued*)

Effect of coumarin derivatives on intracellular levels of H_2O_2 in the wild type euriblastoma cells induced by H_2O_2

H_2O_2 has been considered to mediate Aβ-induced toxicity [60]. To further characterize antioxidative activities of cumerin derivatives, we evaluated the antioxidant activity of the tested compounds in wild type neuroblastoma cells, when the oxidative stress was induced by the presence of hydrogen peroxide. Neuroblastoma cells were co-treated with two different concentrations of H_2O_2 (1 μm and 10 μm and coumarin derivatives 50 μm and 100 μm, respectively.

Figure 5 shows the results for concentrations of 1 μm of hydrogen peroxide. Scavenging free radical activity was observed in compounds 14, 15, 16, 17, 18, 3 and 4. The 'parent' molecule, coumarin, was also found to be active. A higher concentration of hydogen peroxide (10 μm) led to the decrease of the scavenging activity of coumarin derivatives (Figure 6). Again compound 21 seems to be very active. Coumarin derivatives 5, 14 and 18 were found to have significant activity.

Effect of coumarin derivatives on cell viability of wild type N2a cells tested with MTT assay

Wild type (N2a) neuroblastoma cells were exposed to hydrogen peroxide (H_2O_2, 100 μm) for 2 h in the medium. Then the cells were treated with coumarin derivatives in two different concentrations (50 μm and 100 μm) for 24 h. Cells survival was assessed with MTT assay.

In most cases the cell survival rate was concentration-dependent. In some cases the increase of concentration didn't lead to an increase of cell viability.

Compounds 6, 8, 10, 11, 13 and 21 presented wild activity in both concentrations. Compounds 6, 8 and 10 were found to protect ($>70\%$) the n cells in lower concentrations. Compounds 4, 14 and 17 were not found to be able to protect the cells under the experimental conditions (Figure 7).

Figure 3. ROS levels in Δ.9 mutant cells without treatment with no butyric acid. There was no Aβ-expression. Cells were assayed for ROS levels with results expressed as percentage of fluorescence (% DCF) relative to untreated controls (determined as 100%). Mutant cells have been treated with the coumarin derivatives in four different concentrations (10, 50, 100 and 250 μm) for 24 h. Vitamin E was used as a reference compound. Quercetin, a natural flavonoid product, was also used in different concentrations as a reference compound (5 μg/ml: 61.5%, 10 μg/ml: 29% and 50 μg/ml: 11.7%).

Discussion

In our previous studies [46–48], we have tested compounds, 1–12 and 13–20, as free radical scavengers *in vitro*. In the most of the cases their scavenging activity was found to be concentration dependent. Compound 9 (Table I) was found to be the most active with high anti-inflammatory activity in the *in vivo* model of carrageen in rat paw oedema [47]. In the present study, we measured the levels of H_2O_2 in cellular models of Aβ toxicities; the wild type neuroblastoma cells and a transgenic cell line expressing Aβ. The scavenging activities in the cells were found to be low in compounds 2–12 (umbelliferone derivatives), which is consistent with a previous study by Kaneko et al. [45], who reported that umbelliferone (7-hydroxy coumarin, compound 1) containing

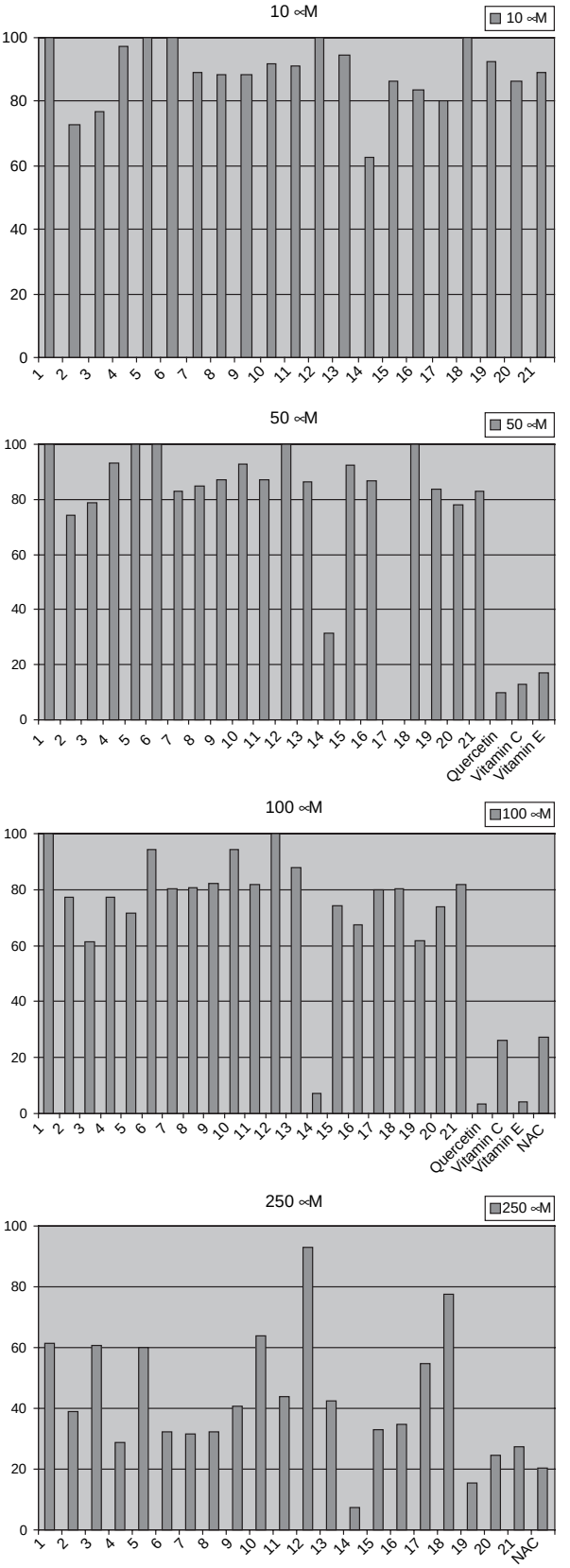


Figure 4. (*Continued*)

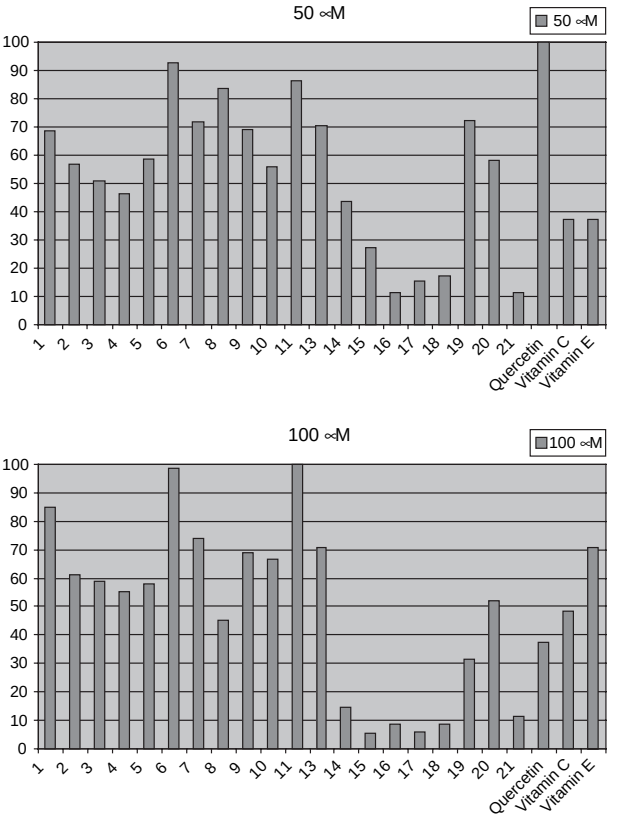


Figure 5. ROS levels in wild type (N2a) neuroblastoma cells with treatment with hydrogen peroxide (H_2O_2: 1 μm) for 2 h. Cells were assayed for ROS levels with results expressed as percentage of fluorescence (% DCF) relative to untreated controls (determined as 100%). Neuroblastoma cells have been treated, after the treatment with hydrogen peroxide, with the coumarin derivatives in two different concentrations (50 and 100 μm) for 24 h. NAC was also used as a reference compound (200 μm: 85.1%).

Figure 4. ROS levels in Δ.9 mutant cells without treatment with butyric acid. There was Aβ-expression. Results are expressed as percentage of fluorescence (% DCF) relative to untreated controls Aβ-secreting cells (determined as 100%). Mutant cells have been treated with the coumarin derivatives in four different concentrations (10, 50, 100 and 250 μm) for 24 h. Quercetin, a natural flavonoid product, was used in different concentrations as a reference compound. Vitamin C, Vitamin E and NAC have been also tested under the same experimental conditions.

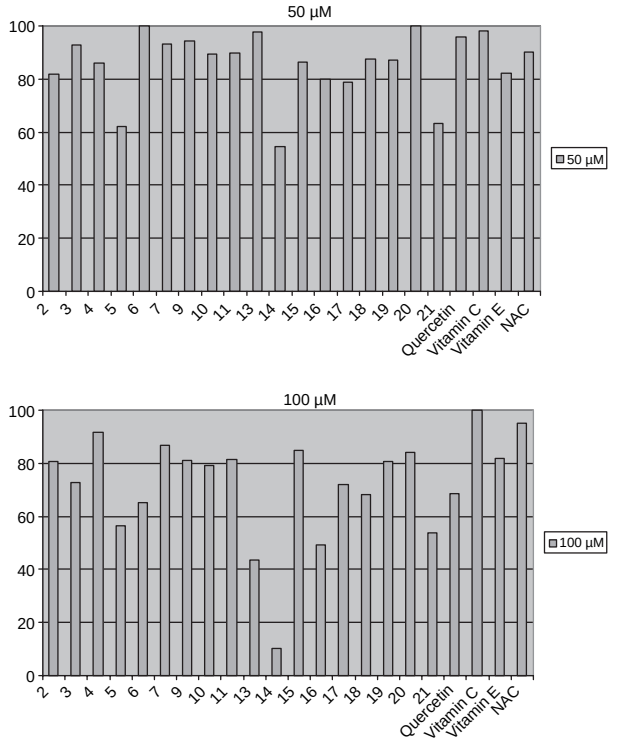


Figure 6. ROS levels in wild type (N2a) neuroblastoma cells with treatment with hydrogen peroxide (H_2O_2: 10 µm) for 2 h. Cells were assayed for ROS levels with results expressed as percentage of fluorescence (% DCF) relative to untreated controls (determined as 100%). Neuroblastoma cells have been treated, after the treatment with hydrogen peroxide, with the coumarin derivatives in two different concentrations (50 and 100 µm) for 24 h.

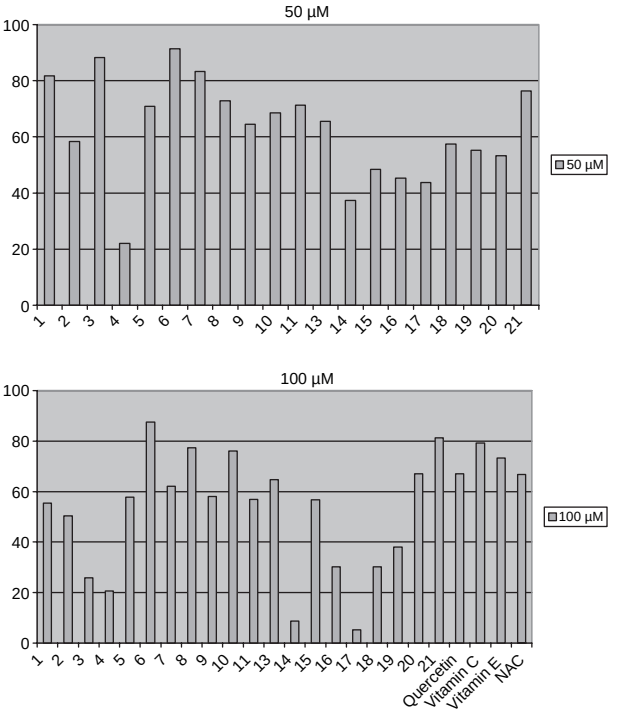


Figure 7. Effects of hydrogen peroxide on wild type (N2a) neuroblastoma cells on cell viability. Human neuroblastoma cells were exposed to two different concentrations of H_2O_2 for 2 h. Cells were then treated for 24 h with the coumarin derivatives or other reference compounds. Cells were treated with two different concentrations of coumarin derivatives (50 and 100 µm). Cell viability was measured by MTT right after the Quercetin, a natural flavonoid product, was used in different concentrations as a reference compound. Vitamin C, Vitamin E and NAC were used as reference compounds. The points represent the mean (±SEM) calculated from three different values.

only one hydroxyl group showed no protective effect in pretreatment or concurrent treatment from injury by linoleic acid hydroperoxide. All the compounds indicated poor results as ROS scavengers.

Compound 8 was also tested for time-dependent scavenging activity and no scavenger activity was found at 6, 12 or 24 h (Figure 2) and at 3 h treatment time the activity is even lower than the untreated controls.

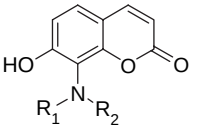
The second series of coumarin derivatives, compounds 13–20, were found to be more active as free radical scavengers than that of the first group (Figure 1). Among them, compounds 16 and 18 presented significant higher free radical scavenging activity compared with the *in vitro* tests previously conducted [46,48]. Compound 16 showed a time dependent free radical scavenging activity in the neuroblastoma cells (Figure 2). Higher scavenging activity was found after 24 h treatment but not 3, 6 or 12 h. It seems that in the cells a 24 h period is needed for compound activity. For compound 14 the radical scavenging response was almost the same at 6, 12 and 24 h. A slight decrease was observed at 3 h.

To associate antioxidant potential of the coumarin derivatives with Aβ-induced oxidative stress, we next used a transgenic neuronal cell line secreting Aβ as a cellular model of AD. Earlier evidence has been provided of elevated ROS in these neuronal cells upon the transgenen Aβ expression [22]. By several research groups a relationship has been investigated between free radical production and Aβ by exposing neuronal cultures to exogenous synthetic $A\beta_{(1-42)}$ [22]. It has been previously shown that Aβ induces the production of hydrogen peroxide and lipid peroxide in neurons [43].

We found that various coumarin derivatives exhibited a profound reduction of hydrogen peroxide (H_2O_2) free radical levels in cell cultures (Figures 5 and 6). In the mutant cells without butyric acid to express the transgen, the first group compounds, 2, 4, 6, 10, 11 and 12, were found to be significantly active. The second group of coumarin derivatives, compounds 16, 17, 18 and 20, were found to be equally active even in lower concentrations. These results are close to the scavenging activity shown in the wild type neuroblastoma cells (Figure 3).

When the mutant cells were treated with butyric acid to express Aβ transgen, the scavenging activities of the coumarin derivatives were found to be concentration-dependent. Concentration increase led to the increase of attenuating ROS levels. Compounds

Table I. Chemical data of coumarin derivatives referring to Scheme 1.



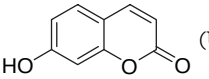
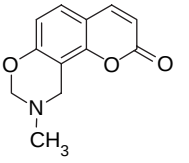
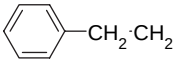
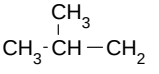
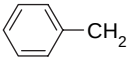
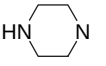
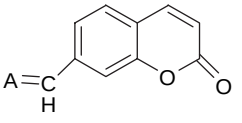
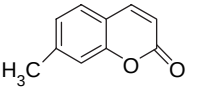
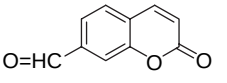
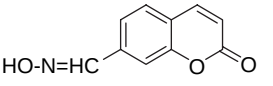
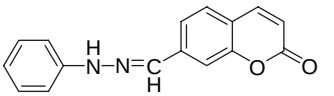
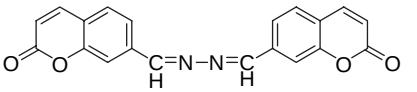
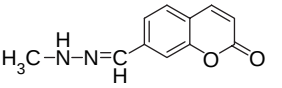
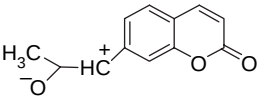
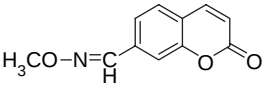
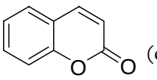
Compounds	Structures		Clog P^{a}	CPE (%)[b,c]
1	 (Umbelliferone)		1.62	no
2			1.37	53.6**
	R_1	R_2		
3			2.60	47.4*
4		H	3.04	31.2*
5	CH_3-CH_2-CH_2-CH_2-CH_2	H	3.48	52.7*
6		H	2.82	55.4*
7		H	3.22	47.5**
8	CH_3-CH_2	H	1.47	53.6**
9			1.11	77.7*
10			1.32	75.7*
11	NH_2-CH_2-CH_2	H	0.76	56.0*
12	H_2N–CH_2-CH_2-CH_2	H	0.42	53.4*
				
13			1.13	55.1
14			1.13	29.3
15			1.62	58.6

Table I (*Continued*)

Compounds	Structures	Clog P^a	CPE (%)[b,c]
16		2.91	35.2
17		0.39	54.0[c]
18		1.00	54.7
19		−0.41	18.0
20		1.51	31.1
21	 (coumarin)		

[a]Theoretically calculated lipophilicity as Clog *P* values; [b]*In vivo* anti-inflammatory test, carrageenin-induced edema at the right hind paw; Intraperitoneally administration of the compounds and the dose used: 0.01 mmoles/Kg body weight; [c]Indomethacin as a standard 47% (0.01 mM).

2–12 were found to have scavenging activity under the experimental conditions (Figure 4). Compounds 2 and 3 presented significant free radical scavenging activity even at lower concentration (10 μm). The coumarin derivatives present differences in scavenging activity between the two types of cells, neuroblastoma and the mutant cells. Compounds 2 and 3 did not show antioxidant activity in wild type neuroblastoma cells. This could be attributable to a selective scavenging activity to mutant cells, which can probably explain the above scavenging activity and could be more interesting as therapeutic agents.

Compounds 13–20 were found to be very active, compound 14 was the most active (Figure 5). According to our findings, the above compounds were found to be very active in the neuroblastoma cell cultures, which show that they present antioxidant activity in both cell cultures.

The role of hydrogen peroxide as a free radical has been already described. To provide additional evidence, we studied the neuroblastoma cells co-treated with coumarin derivatives and hydrogen peroxide (1 and 10 μm) (Figures 6 and 7).

Compounds 2–12 seem to present adequate free radical scavenging activity in neuroblastoma cell cultures treated with hydrogen peroxide. The natural product umbelliferone decreased the ROS level in wild type cells (70–80%). Compound 9 herein seems to present weaker scavenging activity.

Compounds 13–20 were found to present significantly high free radical scavenging activity. Compound 14 was found to be very active in the treatment with hydrogen peroxide 10 μm, but when the hydrogen peroxide concentration was decreased the scavenging activity was also decreased. Compounds 16 and 17 were found to be the most active in both concentrations. The radical scavenging activity of compound 16 was similarly high to all the previous studies.

MTT assay for testing cell viability shows that some of the coumarin derivatives protect against hydrogen peroxide-induced cell death. In previous studies fraxin and other natural coumarin derivatives showed protective effects against hydrogen peroxide oxidative stress [61]. It has been also noticed that when human endothelial cells were pre-treated with fraxetin and other coumarins a significant increase in cell survival was observed [62]. The high protective role of coumarin, (compound 21) and umbelliferone, (compound 1), in other human cell lines is in accordance to previous studies [63].

In our experimental conditions, using the wild type (N2a) neuroblastoma cells, some compounds were found to be protective and their activity was very close to the reference compounds, vitamin C and vitamin E and in some cases even higher. Compounds 2–12 (the umbelliferone derivatives) were found to better protect cell culture than the group of compounds 14–21, with the azomethine linkage at position 7 of the coumarin ring.

Compounds 6, 8 and 10 were found to be more active and they showed the highest protective activity in cell viability. Compound 9 showed an adequate protection to cell cultures. Coumarin derivatives 14–21 showed very low protection to cells. Most of the compounds showed lower protection with the increase of concentration, which should be subject for further investigation.

Conclusions

There is overwhelming evidence that a state of chronic inflammation exists in affected regions of the AD brain. Multiple drug administration, targeted at different inflammatory mechanisms, may prove far more effective than utilization of any single agent [22,23,60].

The presented coumarin derivatives have shown significant anti-inflammatory and antioxidant properties [46,48]. The results of this study demonstrate the abilities of some coumarin derivatives to attenuate elevated levels of ROS with effective and significant attenuation in both wild type and AD-associated transgenic cellular models. The results lead to the conclusion that coumarin hydrazone derivatives could be used for further design of compounds with potent antioxidant and anti-inflammatory activity.

Compounds 9, 16 and 18 can be probably used for further investigation in other experiments in Alzheimer's disease. Their significant *in vivo* anti-inflammatory activity in comparison to the *in vitro* antioxidant activity in neuroblastoma cells and in mutant cells could be quite promising for further research. The high free radical scavenging activity of the parent molecule, compound 21, is mostly important and ensures our first thought that coumarin ring as a basic scaffold could be of high importance for further design of active compounds.

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