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SHORT COMMUNICATION

Further *in vitro* biological activity evaluation of amino-, thio- and ester-derivatives of avarol

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Abstract

The acetylcholinesterase inhibitory and/or antitumour activities of amino-, thio- and ester-derivatives of avarol selected were evaluated for the first time at *in vitro* conditions. Avarol-3′,4′-dithioglycol (**1**) and avarol-4′-(3)mercaptopropionic acid (**3**) were shown to be the best inhibitors of the enzyme tested (0.50 μg and IC_{50} 0.05 mM and 0.50 μg and IC_{50} 0.12 mM, respectively), while 4′-tryptamine-avarone (**9**) and avarol-3′-(3)mercaptopropionic acid (**2**) exhibited the highest cytotoxicity against the human breast T-47D cancer cell line (IC_{50} 0.66 μg/mL and 1.25 μg/mL, respectively). According to experimental data obtained, the sesquiterpenoid hydroquinone structure of bioactive avarol derivatives may inspire development of new pharmacologically useful substances to be used in the treatment of Alzheimer's disease and/or human breast tumour.

Keywords

Acetylcholinesterase inhibitory activity, antitumour activity, *Dysidea avara*, sesquiterpenoid hydroquinone derivatives

History

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Introduction

Avarol and avarone, two sesquiterpenes (hydroquinone and quinone, respectively) possessing a rearranged drimane skeleton, were isolated from the marine sponge *Dysidea avara* Schimdt[1,2] (Figure 1). Previous studies have revealed that these secondary metabolites show a wide variety of biological activities such as antibacterial, antifungal, antiviral, cytotoxic, antioxidant, anti-inflammatory and anti-psoriatic effects[3,4]. Their effect on radical production and their redox chemistry have shown to be involved in biological activities of these compounds[5]. Recent findings indicate that some thio-avarol derivatives exhibit acetylcholinesterase (AChE) inhibitory activity[6]. The abnormal activity of this enzyme is one factor responsible for Alzheimer's disease, the most common cause of senile dementia in later life[7]. The multiple pharmacological properties of avarol, avarone and/or their derivatives prompted us to continue with the *in vitro* screening of the bioactivity noted focusing on AChE inhibitory and antitumour effects. Thio-avarol derivatives represent the majority of compounds tested; however, some amino- and ester-avarol derivatives have been included in the aforementioned screening as well. The synthesis of all derivatives screened has been already known from literature[6,8,9].

Materials and methods

Synthesis of compounds

Thio-, amino- and ester-avarol derivatives were synthesised as previously described[6,8,9].

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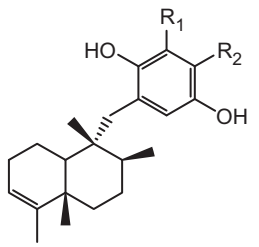
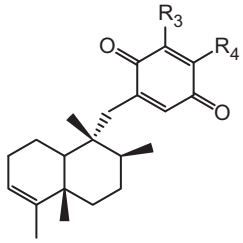
Acetylcholinesterase inhibitory activity

A preliminary AChE inhibitory activity was assessed according to Marston et al.[10]. All compounds tested were assayed at four different concentrations (0.1, 0.5, 1 and 10 μg, respectively) referring to a standard galanthamine (1, 0.1 and 0.01 μg, respectively). The test was performed by dissolving the compounds in MeOH or DMSO, according to their solubility, at a concentration of 1 mg/mL. From this main solution, a serial dilution was performed to obtain lower concentrations of the compounds tested (0.1, 0.01 and 0.001 mg/mL, respectively). On the other hand, a stock solution of AChE (1000 U in 150 mL of Tris-hydrochloric acid buffer pH 7.8) was stabilised by adding bovine serum albumin (150 mg). A 10 μL aliquot of each solution of the samples was applied to the TLC plates, dried to remove the solvent and then sprayed with enzyme stock solution. For incubation of the enzyme, the plate was kept at 37 °C for 20 min in a humid atmosphere. For the detection of the enzyme, solutions of 1-naphthyl acetate (250 mg in 100 mL of EtOH) and Fast Blue B salt (400 mg in 160 mL of distilled H_2O) were mixed and sprayed onto the plate. AChE inhibitory activity was detected by a white spot on a purple background after 1–2 min.

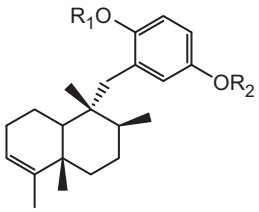
The most active compounds were further tested according the spectrophotometric method of Ellman[11] using AChE (from *Electrophorus electricus*, Sigma-Aldrich, Milan, Italy) and BuChE (from equine serum, Sigma-Aldrich, Milan, Italy). The final volume of reaction was 0.5 mL, containing 0.875 mM of 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) and 0.035 U of AChE or 0.05 U/mL of BuChE, in 0.1 M phosphate-buffered solution pH = 8. The mixture was incubated with eight different concentrations of compounds (from 0.2 μM up to 0.5 mM) for 10 min. After this time, the substrate (0.35 mM AcTCho or 0.5 mM BuTCho) was added to the mixture. The absorbance was





Thio-avarol derivatives
1 R_1=R_2= CH_2OHCH_2S-
2 R_2= H R_1=$C_2H_6O_2S$-
3 R_1=H R_2= $C_2H_6O_2S$-
4 R_1=R_2= $C_2H_6O_2S$-

Amino-avarol derivatives
5 R_3=NHMe R_4=H
6 R_3=H R_4= -HNCH(COOH)CH_2-Ph-
7 R_3= $C_6H_5CH_2NH_2$ R_4=H
8 R_3=H R_4= NHCH(COOH)$CH_2CH(CH_3)_2$
9 R_3= H R_4= tryptamine



Ester-avarol derivative
10 R_1=R_2= *p*-anisoyl-benzoyl-

Figure 1. Chemical structural formula of bioactive avarol derivatives.

Table 1. *In vitro* bioactivity of selected avarol derivatives.

Avarol derivatives	AChE inhibitory activity (μg)*	Antitumour activity (IC^{50}, μg/mL)†
Amino-derivatives		
3′-Methylamino-avarone	1.00	n.a.‡
4′-Phenylanino-avarone	1.00	n.a.
3′-Benzylamine-avarone	1.00	n.a.
4′-Leucine-avarone	n.a.	1.60
4′-Tryptamine-avarone	n.a.	0.66
Thio-derivatives		
Avarol-3′-thiolactic acid	1.00	n.a.
Avarol-4′-thiolactic acid	1.00	n.a.
Avarol-3′-thiosalicic acid	1.00	n.a.
Avarol-3′-(3)mercaptopropionic acid	0.50	1.25
Avarol-3′,4′-dithioglycol	0.50	2.50
Avarol-4′-(3)mercaptopropionic acid	0.50	n.a.
Avarol-3′,4′-(3)mercaptopropionic acid	0.50	n.a.
Ester-derivative		
Di-*p*-anisoyl-benzoyl-avarol	1.00	n.a.

*Acetylcholinesterase (AChE) inhibitory activity expressed as the minimum amount of compound tested that inhibits the enzyme in a TLC bioautographic assay.
†Antitumour activity against the human breast T-47D cancer cell line.
‡n.a. – no activity.

registered at 405 nm in a spectrophotometer plate reader (TECAN GENios PRO, San Jose, CA). Galanthamine was used as a standard, while the mixture without compounds tested was used as a control (100% enzyme activity). The results are expressed as IC_{50} values, i.e. in the form the concentration at which the 50% of enzyme inhibition was observed.

Cell line and culture conditions

The human breast T-47D cancer cell line was cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal calf serum, 1 mM pyruvate, 10 mM HEPES, 100 U/mL penicillin G sodium and 100 mg/mL streptomycin sulfate at 37 °C under 5% CO_2. All media and cell culture supplements were purchased from Gibco–Invitrogen (Carlsbad, CA). The cells (3×10^5) were seeded in 24-well plates and grown for 24 h before the treatment with the compounds tested for the indicated period.

MTT viability assay

The cytotoxicity was evaluated on T-47D cells according to Mosmann et al.[12], using the MTT [3-(4,4-dimethhylthiazol-2-yl)-2,5-phenyl-2H-tetrazolium bromide] method; the percentage of mortality caused by the compounds tested was calculated at three different concentrations (100, 10 and 1 μg/mL). The cells were plated in 96 culture wells at a density of 250 000 cells/mL and allowed to adhere for 2 h. Then medium was replaced with a fresh one and the cells were incubated with the samples (100, 10 and 1 μg/mL, respectively). After 24 h, mitochondrial respiration, an indicator of cell viability, was assessed by the mitochondrial-dependent reduction of MTT to formazan. Briefly, 25 mL of MTT (5 mg/mL in complete DMEM) was added to the cells and incubated for an additional 3 h. After this time point, the cells were lysed and the dark blue crystals were solubilised in 100 mL of a solution containing 50% (v:v) *N,N*-dimethylformamide and 20% (w:v) sodium dodecyl sulfate with an adjusted pH of 4.5. The optical density (OD) of each well was measured with a microplate spectrophotometer (TECAN GENiOS PRO) that was equipped with a 620 nm filter. The cell mortality was calculated in IC_{50} (μg/mL), i.e. as the inhibitory concentration at which 50% of mortality was observed.

Results and discussion

Among all compounds tested in the preliminary AChE inhibition assay, avarol-3′,4′-dithioglycol (**1**), avarol-3′-(3)mercaptopropionic acid (**2**), avarol-4′-(3)mercaptopropionic acid (**3**) and avarol-3′,4′-(3)mercaptopropionic acid (**4**) showed that the highest AChE inhibitory activity (0.50 μg), expressed as the minimum amount of compound at which the enzyme was inhibited. In addition, 3′-methylamino-avarone (**5**), 4′-phenylanino-avarone (**6**), 3′-benzylamine-avarone (**7**) and di-*p*-anisoyl-benzoyl-avarol (**10**) were active at 1 μg (Table 1). Previous screening of AChE inhibitory activity indicated a moderate activity (1 μg) for all thio-avarol derivatives with a carboxylic acid group in the molecule[6]. In comparison, the alkaloid galanthamine used clinically for the treatment of Alzheimer's disease inhibited the enzyme at 0.01 μg. On the basis of these results, the most active compounds (thio-avarol derivatives **1**–**4**) were further tested using Ellman's method (Table 2). Among them, avarol-3′,4′-dithioglycol (**1**) showed the best inhibitory activity with a IC_{50} value of 0.05 mM both on AChE and BuChE, while the compounds **2** and **3** exhibited a good AChE inhibitory activity showing IC_{50} values of 0.14 and 0.12 mM, respectively. It's worth noting that all these three thio-avarol derivatives were more active against AChE than galanthamine (IC_{50} 0.24 mM), unlike the compound **4** which showed to be the least active one (IC_{50} >10 mM). In addition, the compounds **2** and **3** exhibited a considerable inhibitory activity on BuChE with IC_{50} values of 0.19 and 0.06 mM, respectively. However, galanthamine showed to be more effective toward BuChE (IC_{50} 0.02 mM) than all four thio-avarol derivatives tested. Taken all together, these findings confirm the relevance of avarol derivatives as new possible generation of AChE inhibitors pointing out the importance of polar substituents in the hydroquinone ring. Since avarol derivatives do not belong to the class of alkaloids well known both for the inhibition of the enzyme and following side effects, there is a possibility to be found novel satisfactorily active AChE chemicals with less side effects.

The cytotoxicity of all 13 avarol derivatives was evaluated against the human breast T-47D cancer cell line. At the lowest concentration applied (1 μg/mL), the thio derivatives avarol-3′,4′-dithioglycol (**1**) and avarol-3′-(3)-mercaptopropionic acid (**2**)

Table 2. AChE and BuChE inhibitory activities of selected thio-avarol derivatives.

Thio-avarol derivatives	IC_{50} (mM) AChE*	BuChE*
Avarol-3′,4′-dithioglycol	0.05	0.05
Avarol-3′-(3)mercaptopropionic acid	0.14	0.19
Avarol-4′-(3)mercaptopropionic acid	0.12	0.06
Avarol-3′,4′-(3)mercaptopropionic acid	>10	>10
Galanthamine	0.24	0.02

*Acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) inhibitory activities.

exhibited a good activity (IC_{50} 2.50 and 1.25 μg/mL, respectively). On the other hand, the amino derivatives 4′-leucine-avarone (**8**) and 4′-tryptamine-avarone (**9**) showed even better cytotoxic effects (IC_{50} 1.60 and 0.66 μg/mL, respectively); no any cytotoxicity was observed for the ester-avarol derivative (Table 2). Avarol and avarone are known for their cytotoxicity evaluated by the brine shrimp test showing a LD_{50} of 0.18 and 0.14 ppm, respectively[13]. Indeed, antitumour activity of these both natural products and some their derivatives have been evaluated on a panel of cancer cell lines *in vitro* and *in vivo* so far[14–16]. The results reported herein for the first time suggest that amino-avarol derivatives may inspire novel leads against the breast T-47D cancer cell line.

Conclusions

The further research lead by comprehensive structure–activity relationship studies may offer new thio- and amino-avarol derivatives with improved properties related to Alzheimer's disease and/or human breast tumour[17].

Declaration of interest

The authors report no declarations of interest.

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