Journal of the Iranian Chemical Society (2021) 18:213–224
https://doi.org/10.1007/s13738-020-02018-6

ORIGINAL PAPER

Novel nicotinonitrile-coumarin hybrids as potential acetylcholinesterase inhibitors: design, synthesis, in vitro and in silico studies

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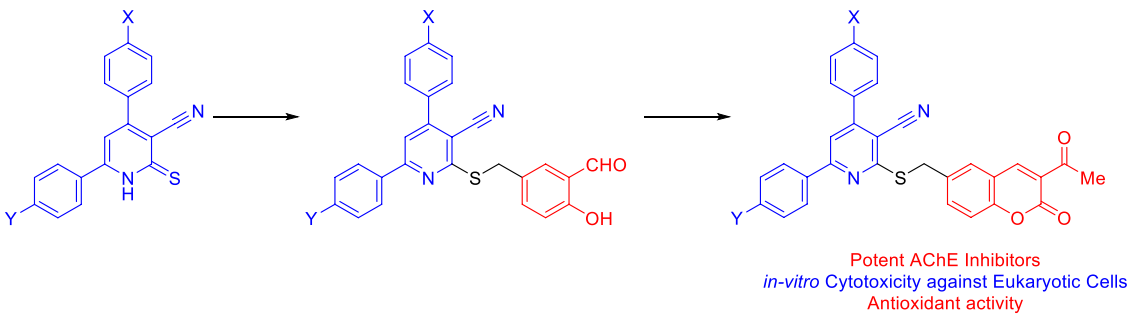
Received: 19 May 2020 / Accepted: 22 July 2020 / Published online: 30 July 2020

Abstract

Alzheimer's disease is a degenerative brain condition that is the leading cause of dementia affecting millions of people around the world. Therapeutic development has focused on the problem of the loss of basal forebrain cholinergic function, as it is the only evidence responsible for brain neurodegeneration in patients with Alzheimer's disease. Several attempts to improve cholinergic neurotransmission have been investigated by minimizing synaptic degradation of acetylcholine using acetylcholinesterase inhibitors. In the current study, we explore the designing of a new series of nicotinonitrile-coumarin hybrids as potential acetylcholinesterase inhibitors. The new hybrids were prepared utilizing pyridine-2(1*H*)-thiones as starting precursors. The in vitro acetylcholinesterase (AChE) inhibitory activities were examined for the new nicotinonitrile-coumarin hybrid molecules, when compared with donepezil as a standard drug with IC_{50} of 14 nM. Coumarin derivative, linked to 6-(4-nitrophenyl)-4-phenylnicotinonitrile, showed more effective inhibitory activity than the reference donepezil with IC_{50} of 13 nM. The free radical-scavenging capabilities against DPPH of the new hybrid derivatives were screened. Additionally, their in vitro cytotoxic activities have been tested against various eukaryotic cells. Furthermore, docking study showed excellent interaction between nicotinonitrile-coumarin hybrids and AChE.

Graphic abstract



Keywords Alzheimer's disease · Nicotinonitrile-coumarin hybrids · Acetylcholinesterase inhibitors · In vitro cytotoxicity screening · In silico study

Introduction

Alzheimer's disease (AD) is a progressive and degenerative brain condition that primarily affects older people and induces memory loss and the ability to perform normal activities. AD is the leading cause of dementia affecting about 50 million people worldwide, plus about

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10 million new patients per year. AD contributes 60–70% of dementia cases [1–3].

However, the true nature or cause of AD remains unclear, and therefore, the development of an effective anti-Alzheimer's is one of the promising fields of current research in medicinal chemistry [4]. Over the past two decades, numerous studies of anatomy within the hippocampus, which relates to learning and perception, have revealed that neurotransmitter acetylcholine levels in patients with AD decrease significantly [5–7]. Nowadays, therapeutic development has focused on the problem of loss of cholinergic function of the basal forebrain, since it is the only evidence responsible for cognitive decline and neurodegeneration in the brains of AD patients [8–10].

Many approaches have been explored to improve cholinergic neurotransmission such as enhanced acetylcholine level (ACh) synthesis or pre-synaptic release and stimulation of postsynaptic muscarinic and nicotinic receptors, as well as to minimize ACh synaptic degradation using acetylcholinesterase (AChE) inhibitors [11–18]. AChE inhibitors approved for treatment with AD are currently donepezil, tacrine, galantamine and rivastigmine (see Fig. 1) [19].

Coumarins are natural heterocyclic derivatives used as essential components in the inhibition and cure of numerous diseases. These derivatives act as antimicrobials, [20] anti-inflammatory, [21] anticancer, [22] anti-Alzheimer's disease [23, 24] and anti-Parkinson agents [25]. Examples of chromene derivatives considered to be effective oral anticoagulants include warfarin and dicoumarol [26]. In addition, coumarin moiety has been included in various effective AChE inhibitors [27–30].

In connection with our efforts to prepare heterocyclic derivatives of potent biological activities [31–36], we report herein the synthesis of novel series of nicotinonitrile-coumarin hybrid molecules, bearing arene moieties and linked via thioethers. The new compounds were subjected to in vitro as well as in silico study to shade the light on their capability as potential acetylcholinesterase inhibitors.

Results and discussion

Chemistry

Recently, we reported the synthesis of new nicotinonitrile-coumarin hybrid molecules as potent antibacterial and anticancer agents [37]. Encouraged by these results, we report herein the synthesis of new coumarin derivatives linked to nicotinonitriles via thioethers as potential acetylcholinesterase inhibitors.

Our first step toward our target was the preparation of numerous nicotinonitriles. We are investigating the synthetic potential of pyridine-2(1*H*)-thiones as effective examples of nicotinonitriles in this study. Both benzaldehyde **1a** and anisaldehyde **1b** were reacted with a series of 4-substituted acetophenones **2a–2e** in ethanolic potassium hydroxide solution under stirring at room temperature for 3 h to give the corresponding α,β-unsaturated carbonyl compounds **3(4)** [38–42]. The starting precursors pyridine-2(1*H*)-thione derivatives **6(7)** were prepared by the cyclocondensation of **3(4)** with 2-cyanothioacetamide **5** in ethanol in the presence of piperidine as a catalyst at reflux for 5 h [43–45] (Scheme 1).

Next, a new series of 2-hydroxybenzaldehydes **9(10)**, bearing nicotinonitrile moieties, was prepared in excellent yields. Thus, each of pyridine-2(1*H*)-thiones **6(7)** was stirred with an equivalent amount of potassium hydroxide in ethanol at room temperature for 10 min. Then, an equivalent amount of 5-(chloromethyl)-2-hydroxybenzaldehyde **8** [46] was added with continuous stirring for 2 h to afford the corresponding 2-hydroxybenzaldehydes **9(10)** in 88–94% yields (Scheme 2). The structures of the isolated products **9(10)** were confirmed via their elemental analyses and spectral data. The IR spectrum of **10a**, as a representative example, revealed the presence of OH, CN and CO groups at 3437, 2233 and 1675 cm^{-1}, respectively. Its mass spectrum gave a molecular ion peak at $m/z = 452$. Its ^{1}H-NMR spectrum revealed five singlet signals at δ 3.85, 4.66, 7.87, 10.20 and 10.68 attributed to OCH_3, SCH_2, pyridine-H5, CHO and OH protons, respectively. In addition, it showed 12 aromatic protons in the range of 6.95–8.27. Its ^{13}C-NMR spectrum

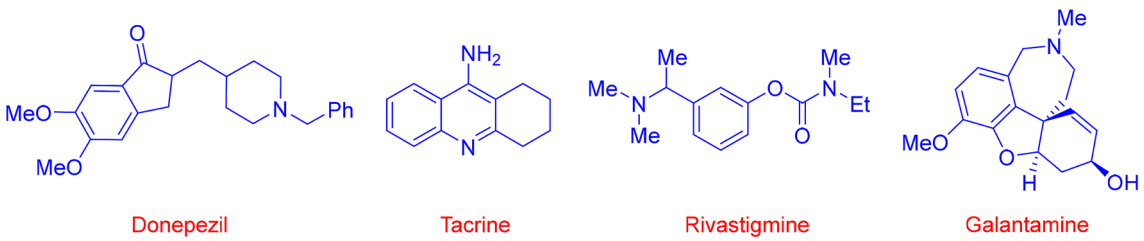
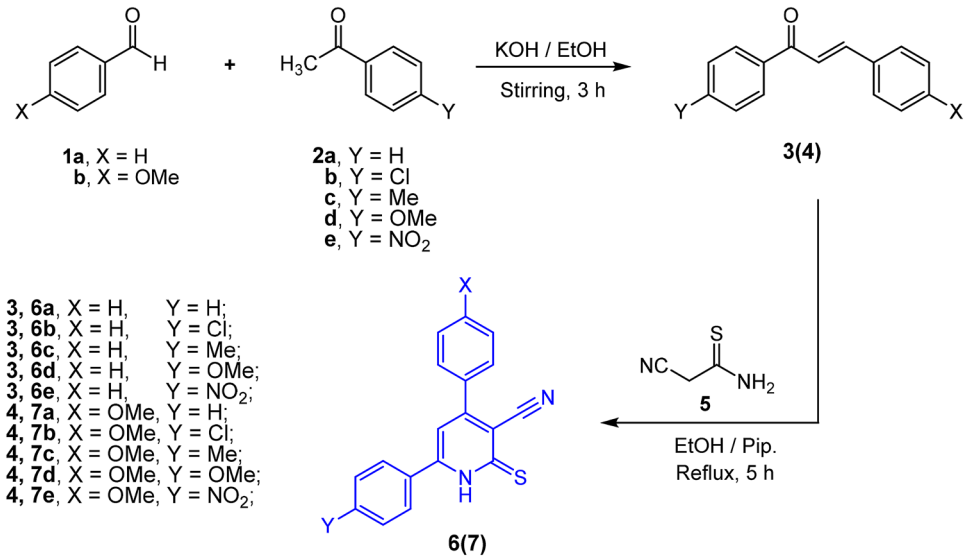


Fig. 1 Structure of AChE inhibitors approved for treatment of AD

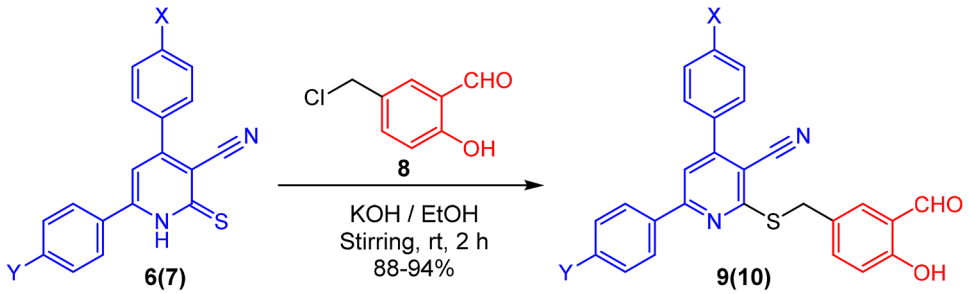
showed the presence of five signals at δ 33.4, 55.9, 115.8 and 191.6 assigned to SCH_2, OCH_3, CN and C=O carbons, respectively, in addition to 19 signals corresponding to pyridine and aromatic carbons (see experimental section).

Subsequently, Knoevenagel synthesis of novel coumarins **12(13)** was carried out using 2-hydroxybenzaldehydes **9(10)** and ethyl acetoacetate **11** in ethanol in the presence of piperazine citrate (1:1, 10 mol%) at 80 °C for 3 h to give the corresponding 2-(((3-acetyl-2-oxo-2*H*-chromen-6-yl)methyl) thio)nicotinonitriles **12(13)** in 84–90% yields (Scheme 3) [37]. The structures of **12(13)** were confirmed via their elemental analyses and spectral data. The IR spectrum of **12d**,

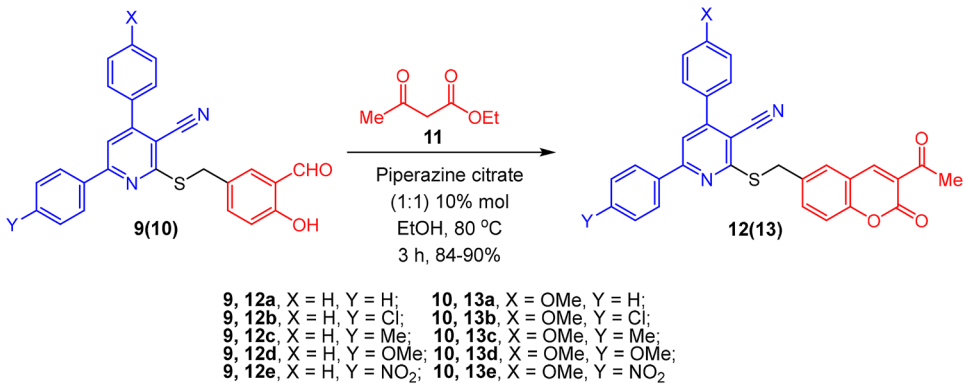
Scheme 1 Synthesis of pyridine-2(1*H*)-thione derivatives **6(7)**



Scheme 2 Synthesis of 2-hydroxybenzaldehydes **9(10)**



Scheme 3 Synthesis of nicotinonitrile-coumarin hybrids **12(13)**



as a representative example, showed the presence of CN and two CO groups at 2233, 1720, 1676 cm^{-1}, respectively. Its mass spectrum gave a molecular ion peak at $m/z = 518$. The ^{1}H-NMR spectrum of **12d** revealed four singlet signals at δ 2.55, 3.85, 4.76 and 7.87 assigned to $COCH_3$, OCH_3, SCH_2 and pyridine-H5 protons, respectively. Moreover, it reveals two doublet signals at δ 7.40 and 7.84 and two singlet signals at δ 8.04 and 8.50 corresponding to four chromene-H8, H7, H5 and H4 protons, respectively. Its ^{13}C-NMR spectrum revealed the presence of five signals at δ 30.4, 33.4, 55.3, 116.8 and 195.1 corresponding to CH_3, SCH_2, OCH_3, CN and C=O carbons, respectively, in addition to 22 signals corresponding to pyridine, aromatic and chromene carbons (see experimental section).

Biology

The in vitro AChE inhibitory activity

The in vitro AChE inhibitory activities were estimated for the new nicotinonitrile-coumarin hybrid molecules **12a–12e** as well as **13a–13e** using Ellman method [47]. The inhibitory activity findings are expressed as the concentration of the tested derivative **12(13)** needed to inhibit 50% of AChE enzyme (IC_{50}, nM). Donepezil was used as a standard drug with IC_{50} of 14 nM [48]. The findings are listed in Table 1.

Among the series **12a–12e**, bearing 4-phenyl group at nicotinonitrile moiety, compound **12e**, with *p*-NO_2 linked to 6-aryl moiety, exhibited the most effective AChE inhibitory activity. Thus, **12e** showed more potent inhibitory activity than the reference donepezil with IC_{50} of 13 nM. Compound **12b**, linked to 6-(4-chlorophenyl) group, was the second in inhibitory strength toward AChE enzyme. Compound **12b** showed IC_{50} of 25 nM when compared with donepezil. Other hybrid derivatives **12a**, **12c** and **12d** exhibited decreased activities toward AChE. Therefore, compound **12a**, linked to 6-phenyl group, showed IC_{50} of 767 nM. On the other hand, compounds **12c** and **12d**, with *p*-Me and *p*-OMe linked to 6-aryl moiety, respectively, exhibited the least potent activities with IC_{50} of 1162 and 1544 nM, respectively.

Regarding the series **13a–13e**, bearing 4-(4-methoxyphenyl) group at nicotinonitrile moiety, this series exhibited generally less effective inhibitory activities toward AChE enzyme. Furthermore, the inhibitory activities of the hybrid derivatives examined were observed in the same order as in the series **12a–12e**. Thus, compounds **13b** and **13e**, with *p*-Cl and *p*-NO_2 linked to 6-aryl moiety, respectively, exhibited the best AChE inhibitory activities with IC_{50} of 324 and 226 nM, respectively. Other hybrid derivatives **13a**, **13c** and **13d**, with *p*–H, *p*-Me and *p*-OMe linked to 6-aryl group, respectively, exhibited the least activities with IC_{50} values in the range of 1762 and 2338 nM.

In order to elucidate the relationship between electronic properties of the target nicotinonitrile-coumarin hybrids and their AChE inhibitory activities, we compare the values of electronic substituent constant (σ_p) [49] with the IC_{50} values (Table 2). After examining Table 2, a clear relation is observed between the values of σ_p and IC_{50} values. In each series **12** or **13**, we observed that the AChE inhibitory activity increased as the substituent, linked to 6-aryl moiety at nicotinonitrile, became more electron withdrawal.

DPPH antioxidant activity

The study of the antioxidant activities of the new hybrid compounds is considered to add value for the diagnosis of Alzheimer's disease. Hence, the free radical-scavenging capabilities of the new hybrid derivatives were examined against DPPH (1,1-diphenyl-1-picrylhydrazyl). Ascorbic acid was used as a reference drug, and the findings are shown in Table 3. Compounds **12b** and **12e** exhibited the most effective free radical quenching results in DPPH assay with IC_{50} of 35.4 and 28.5 µM, respectively, when compared with ascorbic acid (IC_{50} of 20.4 µM). Other tested derivatives displayed decreased quenching abilities in DPPH assay with IC_{50} more than 45 µM.

Table 1 The AChE inhibitory activities of new nicotinonitrile-coumarin hybrids as expressed in IC_{50} values (nM)

Cpd	**12a**	**12b**	**12c**	**12d**	**12e**	**13a**	**13b**	**13c**	**13d**	**13e**	**Donepezil**
X	H	H	H	H	H	OMe	OMe	OMe	OMe	OMe	–
Y	H	Cl	Me	OMe	NO_2	H	Cl	Me	OMe	NO_2	–
IC_{50} (nM)	767	25	1162	1544	13	1762	324	2218	2338	226	**14**

Table 2 The relation between electronic substituent constants σ_p and IC_{50} values

Cpd	**12a**	**12b**	**12c**	**12d**	**12e**	**13a**	**13b**	**13c**	**13d**	**13e**
Y	H	Cl	Me	OMe	NO_2	H	Cl	Me	OMe	NO_2
IC_{50} (nM)	767	25	1162	1544	13	1762	324	2218	2338	226
σ_p (Y substituents)	0.0	0.23	−0.17	−0.27	0.78	0.0	0.23	−0.17	−0.27	0.78

Table 3 DPPH scavenging activity of the synthesized compounds

Cpd	**12a**	**12b**	**12c**	**12d**	**12e**	**13a**	**13b**	**13c**	**13d**	**13e**	**Ascorbic acid**
IC_{50} in μM (in μg/mL)	> 45 (> 25)	35.4 (18.5)	> 45 (> 25)	> 45 (> 25)	28.5 (15.2)	> 45 (> 25)	> 45 (> 25)	> 45 (> 25)	> 45 (> 25)	> 45 (> 25)	20.4 (3.6)

Table 4 The cell inhibition percentage and IC_{50} values of some new hybrid derivatives against each of MCF-10A, MCF-7, Caco2 and HEPG2, and using Doxorubicin as a reference

Compound	IC_{50} (μM)			
	MCF-10A	MCF-7	Caco2	HEPG2
12b	11.6 ± 0.2	10.2 ± 0.3	15.9 ± 0.3	10.7 ± 0.3
12e	10.5 ± 0.3	8.6 ± 0.2	7.5 ± 0.2	6.2 ± 0.2
13b	16.8 ± 0.4	18.1 ± 0.3	19.6 ± 0.4	18.4 ± 0.4
13e	14.6 ± 0.3	15.4 ± 0.3	17.8 ± 0.3	14.3 ± 0.3
Doxorubicin	12.7 ± 0.3	12.2 ± 0.2	14.5 ± 0.3	13.7 ± 0.3

Cytotoxicity against eukaryotic cells

New hybrid derivatives **12b**, **12e**, **13b** and **13e** have been reported for in vitro cytotoxic activities against each human breast epithelial cell line (MCF-10A), human breast carcinoma cell line (MCF-7), colon cancer cell line (Caco2) and liver hepatocellular carcinoma cell line (HEPG2) (Table 4). The results of cytotoxicity are expressed as the concentration of the tested derivatives needed to inhibit 50% of cell growth (IC_{50}, μM). Doxorubicin was used as a standard drug with IC_{50} of 12.2 ± 0.2, 12.7 ± 0.3, 13.7 ± 0.3 and 14.5 ± 0.3 μM against each of MCF-7, MCF-10A, HEPG2 and Caco2, respectively (see Table 4).

Derivatives **12b** and **12e** showed more powerful cytotoxic effects against each of the MCF-7, MCF-10A and HEPG2 cell lines than the reference Doxorubicin. Therefore, **12b** and **12e** showed IC_{50} of 6.2–11.6 μM against these cell lines. On the other hand, compounds **13b** and **13e** exhibited decreased cytotoxic activity with IC_{50} of 14.3–18.4 μM relative to Doxorubicin against the above cell lines (Table 4).

With regard to the cytotoxicity against Caco2 cells, compound **12e** exhibited more effective cytotoxic activity than the reference Doxorubicin with IC_{50} of 7.5 ± 0.2 μM. In addition, derivatives **12b**, **13b** and **13e** exhibited decreased activities with IC_{50} of 15.9–19.6 μM, when compared with Doxorubicin (Table 4).

It is worth noting that cytotoxicity of the new hybrids **12b**, **12e**, **13b** and **13e** showed IC_{50} in micromolar range (IC_{50} of 6.2–19.6 μM) against all the cell lines tested, while these hybrids showed effective AChE inhibitory activities in nanomolar range (IC_{50} of 13–324 nM). These findings clearly showed that the new hybrids are not cytotoxic at the concentration needed to effectively inhibit AChE.

The in silico study: molecular modeling

AChE enzyme is expressed in cholinergic neurons and neuromuscular junctions. Its main function is the fast breaking down of the neurotransmitter acetylcholine that is released during cholinergic neurotransmission [50]. Molecular docking was performed to test the capability of some new nicotinonitrile-coumarin hybrids as potential AChE inhibitors. Thus, compounds **12b** and **12e** were docked as ligand molecules with human acetylcholinesterase (PDB ID: 4EY7) as the target protein to achieve their optimum conformation, with reduced free energy [51, 52]. Computational docking studies have been used to estimate the binding energies of the interactions between each ligand and the amino acid residues in the target enzyme [53]. The docking findings of **12b** and **12e** are shown in Table 5.

Both experimental and docking results demonstrated capability of coumarin **12e** as a potent AChE inhibitor. Compound **12e** demonstrated strong AChE binding interactions (PDB ID: 4EY7) as shown in Fig. 2. The figure revealed a strong network of hydrogen bonding interactions of cyano-N, acetyl-O and thioether-S atoms with aminoacids residues of ASN 233 (2.81 Å, − 5.8 kcal/mol), ARG 247 (3.05 Å, − 4.5 kcal/mol) and GLU 313 (3.46 Å, − 2.4 kcal/mol), respectively. In addition, nitro group linked to 6-arylnicotinonitrile moiety showed strong hydrogen bonding interactions with aminoacids residues of both ARG 296 (2.76 Å, − 4.3 kcal/mol; 2.90 Å, − 3.2 kcal/mol) and GLN 369 (3.00 Å, − 2.7 kcal/mol). It showed also strong π–H interactions between coumarin moieties with residue of VAL 239 (2.93 Å, − 4.0 kcal/mol).

Docking results of coumarin **12b** are shown in Fig. 3. The figure revealed a good network of hydrogen bonding interactions of cyano-N and coumarin-O atoms with aminoacids residues of VAL 239 (2.85 Å, − 5.7 kcal/mol) and ASN 233 (3.15 Å, − 4.2 kcal/mol; 3.27 Å, − 3.9 kcal/mol), respectively. In addition, chlorine atom linked to 6-arylnicotinonitrile moiety showed strong hydrogen bonding interactions with aminoacids residues of both PRO 537 (2.82 Å, − 3.6 kcal/mol) and SER 541 (2.96 Å, − 3.3 kcal/mol). It showed also strong π–H interactions between coumarin moieties with residue of GLU 313 (3.06 Å, − 3.7 kcal/mol).

Table 5 Energy values obtained in docking calculations of new nicotinonitrile-coumarin hybrids **12b** and **12e** with AChE (PDB ID: 4EY7)

Compound	Ligand moiety	Site	Interaction	Distance (Å)	*E* (kcal/mol)
12b	CL 54	O PRO 537 (A)	H-donor	2.82	−3.6
	CL 54	OG SER 541 (A)	H-donor	2.96	−3.3
	O 47	CB ASN 233 (A)	H-acceptor	3.15	−4.2
	O 47	ND2 ASN 233 (A)	H-acceptor	3.27	−3.9
	N 56	CA VAL 239 (A)	H-acceptor	2.85	−5.7
	6-ring	CB GLU 313 (A)	Pi-H	3.06	−3.7
12e	S 8	OE1 GLU 313 (A)	H-donor	3.46	−2.4
	O 49	NH2 ARG 247 (A)	H-donor	3.05	−4.5
	N 55	CB ASN 233 (A)	H-acceptor	2.81	−5.8
	O 57	NE ARG 296 (A)	H-acceptor	2.76	−4.3
	O 57	NH2 ARG 296 (A)	H-acceptor	2.90	−3.2
	O 58	CB GLN 369 (A)	H-acceptor	3.00	−2.7
	6-ring	CA VAL 239 (A)	Pi-H	2.93	−4.0

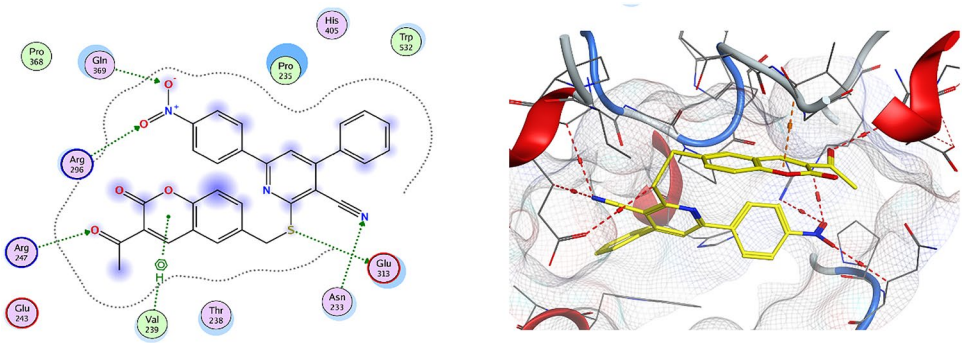


Fig. 2 The 2D and 3D ligand interactions of **12e** with AChE (PDB ID: 4EY7)

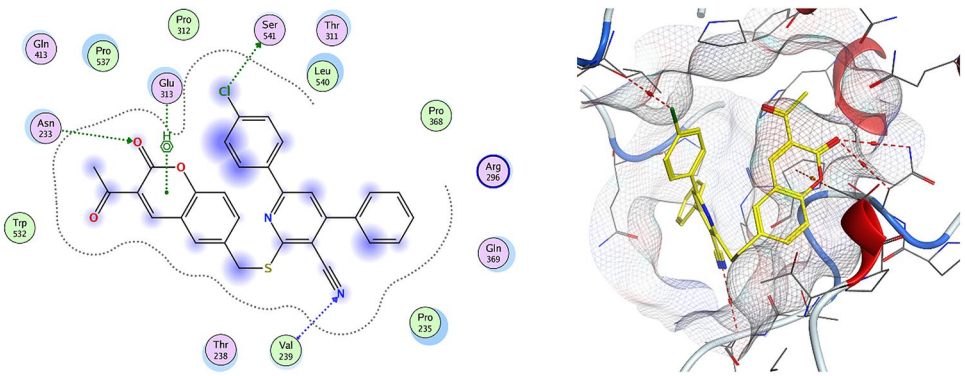


Fig. 3 The 2D and 3D ligand interactions of **12b** with AChE (PDB ID: 4EY7)

Conclusion

The 2-hydroxybenzaldehydes, bearing nicotinonitrile moieties, were prepared in excellent yields and used as starting precursors for the synthesis of a new series of nicotinonitrile-coumarin hybrid derivatives. The in vitro acetylcholinesterase inhibitory activities were examined for the new hybrid derivatives. Coumarin derivative linked to 6-(4-nitrophenyl)-4-phenylnicotinonitrile showed more effective inhibitory activity than the reference donepezil. Some of the new hybrids showed good free radical-scavenging capabilities against DPPH as well as cytotoxic activities against various eukaryotic cells. Docking study predicted the capability of new coumarins as potential AChE inhibitors.

Experimental

Introduction

All solvents were acquired from commercial sources and used as received unless otherwise stated. Piperazine citrate (1:1) was acquired from Boc Sciences (CAS 14396-16-8). All other chemicals were acquired from Merck or Aldrich. These chemicals were used without further purification. The melting points were measured on a Stuart melting point apparatus and are uncorrected. IR spectra were recorded on a Smart iTR, which is an ultra-high-performance, versatile attenuated total reflectance (ATR) sampling accessory on the Nicolet iS10 FT-IR spectrometer manufactured. NMR spectra were recorded on Bruker Avance III 400 MHz spectrophotometer (400 MHz for ^{1}H and 100 MHz for ^{13}C) using TMS as an internal standard and DMSO-d_6 as solvent, and chemical shifts were expressed as δ ppm units. Mass spectra were recorded on a GC–MS-QP1000EX spectrometer using inlet type at 70 eV. Elemental analyses were carried out on a EuroVector instrument C, H, N analyzer EA3000 Series.

General procedures and spectral data

General procedure for the synthesis of 2-((3-formyl-4-hydroxybenzyl)thio)nicotinonitrile derivatives 9(10) A mixture of pyridine-2(1*H*)-thiones **6(7)** (5 mmol) and potassium hydroxide (5 mmol) in ethanol (15 mL) was stirred at room temperature for 10 min. Then, 5-(chloromethyl)-2-hydroxybenzaldehyde **8** was added and the stirring was continued for 2 h. The product was collected by filtration, washed with each of water and cold ethanol, dried and recrystallized from the proper solvent.

2-((3-Formyl-4-hydroxybenzyl)thio)-4,6-diphenylnicotinonitrile (9a) Colorless solid (ethanol, 90%); m.p. 114–115 °C; IR (υ cm^{-1}): 3422 (OH), 2236 (CN), 1679 (CO); ^{1}H-NMR (DMSO-d_6): δ 4.63 (s, 2H, SCH_2), 6.96 (d, 1H, Ar–H), 7.46 (t, 1H, Ar–H), 7.50 (t, 2H, Ar–H's), 7.54 (t, 1H, Ar–H), 7.59 (t, 2H, Ar–H's), 7.65 (d, 1H, Ar–H), 7.73 (d, 2H, Ar–H's), 7.80 (s, 1H, Ar–H), 7.83 (s, 1H, pyridine-H5), 7.95 (d, 2H, Ar–H's), 10.22 (s, 1H, CHO), 10.67 (s, 1H, OH); ^{13}C-NMR (DMSO-d_6): δ 33.7 (SCH_2), 103.9, 115.4, 116.2, 117.7, 122.5, 127.7, 128.0, 128.2, 128.5, 129.1, 129.5, 129.9, 130.5, 134.9, 136.3, 137.1, 152.8, 157.6, 161.2, 162.0, 191.8 (*C*O); Anal. for $C_{26}H_{18}N_2O_2S$ (422.5): C, 73.91; H, 4.29; N, 6.63; found: C, 73.74; H, 4.42; N, 6.50%.

6-(4-Chlorophenyl)-2-((3-formyl-4-hydroxybenzyl)thio)-4-phenylnicotinonitrile (9b) Colorless solid (dioxane/ethanol, 91%); m.p. 120–122 °C; IR (υ cm^{-1}): 3425 (OH), 2230 (CN), 1677 (CO); ^{1}H-NMR (DMSO-d_6): δ 4.64 (s, 2H, SCH_2), 6.96 (d, 1H, Ar–H), 7.50 (d, 2H, Ar–H's), 7.56 (t, 1H, Ar–H), 7.60 (t, 2H, Ar–H's), 7.66 (d, 1H, Ar–H), 7.78 (s, 1H, Ar–H), 7.81 (s, 1H, pyridine-H5), 7.95 (d, 2H, Ar–H's), 8.12 (d, 2H, Ar–H's), 10.22 (s, 1H, CHO), 10.65 (s, 1H, OH); ^{13}C-NMR (DMSO-d_6): δ 33.4 (SCH_2), 103.5, 115.8, 116.4, 117.9, 122.6, 127.8, 128.1, 128.2, 128.9, 129.0, 129.4, 130.6, 135.0, 135.7, 136.2, 137.4, 153.7, 158.7, 161.5, 162.4, 191.9 (*C*O); Anal. for $C_{26}H_{17}ClN_2O_2S$ (456.9): C, 68.34; H, 3.75; N, 6.13; found: C, 68.69; H, 3.51; N, 6.10%.

2-((3-Formyl-4-hydroxybenzyl)thio)-4-phenyl-6-(*p*-tolyl)nicotinonitrile (9c) Colorless solid (dioxane/ethanol, 88%); m.p. 120–121 °C; IR (υ cm^{-1}): 3432 (OH), 2231 (CN), 1675 (CO); δ 2.38 (s, 3H, *p*-CH_3), 4.64 (s, 2H, SCH_2), 6.96 (d, 1H, Ar–H), 7.33 (d, 2H, Ar–H's), 7.54 (t, 1H, Ar–H), 7.59 (t, 2H, Ar–H's), 7.64 (d, 1H, Ar–H), 7.79 (s, 1H, Ar–H), 7.82 (s, 1H, pyridine-H5), 7.96 (d, 2H, Ar–H's), 8.14 (d, 2H, Ar–H's), 10.21 (s, 1H, CHO), 10.65 (s, 1H, OH); ^{13}C-NMR (DMSO-d_6): δ 21.5 (*p*-*C*H_3), 33.6 (SCH_2), 103.3, 116.2, 116.4, 117.8, 122.4, 127.7, 127.8, 128.1, 128.8, 129.1, 129.4, 130.7, 134.6, 136.0, 137.2, 141.3, 154.8, 158.1, 161.3, 162.1, 191.7 (*C*O); Anal. for $C_{27}H_{20}N_2O_2S$ (436.5): C, 74.55; H, 4.69; N, 6.27; found: C, 74.29; H, 4.62; N, 6.42%.

2-((3-Formyl-4-hydroxybenzyl)thio)-6-(4-methoxyphenyl)-4-phenylnicotinonitrile (9d) Colorless solid (dioxane/ethanol, 94%); m.p. 182–184 °C; IR (υ cm^{-1}): 3414 (OH), 2231 (CN), 1674 (CO); ^{1}H-NMR (DMSO-d_6): δ 3.83 (s, 3H, OCH_3), 4.64 (s, 2H, SCH_2), 6.96 (d, 1H, Ar–H), 7.06 (d, 2H, Ar–H's), 7.52 (t, 1H, Ar–H), 7.57 (t, 2H, Ar–H's), 7.62 (d, 1H, Ar–H), 7.71 (s, 1H, Ar–H), 7.78 (s, 1H, pyridine-H5), 7.95 (d, 2H, Ar–H's), 8.07 (d, 2H, Ar–H's), 10.22 (s, 1H, CHO), 10.63 (s, 1H, OH); ^{13}C-NMR (DMSO-d_6): δ 33.5 (SCH_2), 55.3 (OCH_3), 102.8, 114.2, 116.0, 116.7, 118.0, 122.4, 127.7, 128.1, 128.2, 128.8, 129.1, 129.4, 130.7, 136.0, 137.2, 154.8, 158.1, 158.2, 161.3, 162.1, 191.7 (*C*O); MS (*m*/*z*): 452 (M^+, 63.6%); Anal. for $C_{27}H_{20}N_2O_3S$: C, 71.66; H, 4.45; N, 6.19; found: C, 71.52; H, 4.29; N, 6.30%.

2-((3-Formyl-4-hydroxybenzyl)thio)-6-(4-nitrophenyl)-4-phenylnicotinonitrile (9e) Colorless solid (dioxane, 92%); m.p. 132–135 °C; IR (υ cm^{-1}): 3425 (OH), 2230 (CN), 1677 (CO); ^{1}H-NMR

(DMSO-d_6): δ 4.66 (s, 2H, SCH_2), 6.94 (d, 1H, Ar–H), 7.53 (t, 1H, Ar–H), 7.59 (t, 2H, Ar–H's), 7.64 (d, 1H, Ar–H), 7.80 (s, 1H, Ar–H), 7.84 (s, 1H, pyridine-H5), 7.96 (d, 2H, Ar–H's), 8.05 (d, 2H, Ar–H's), 8.38 (d, 2H, Ar–H's), 10.22 (s, 1H, CHO), 10.65 (s, 1H, OH); ^{13}C-NMR (DMSO-d_6): δ 33.9 (S*C*H_2), 104.2, 115.8, 116.5, 117.8, 122.4, 122.8, 124.4, 127.7, 128.2, 129.1, 129.5, 130.7, 136.3, 137.1, 138.5, 144.6, 153.5, 157.5, 161.0, 162.3, 191.5 (*C*O); Anal. for $C_{26}H_{17}N_3O_4S$ (467.5): C, 66.80; H, 3.67; N, 8.99; found: C, 66.88; H, 3.86; N, 8.73%.

2-((3-Formyl-4-hydroxybenzyl)thio)-4-(4-methoxyphenyl)-6-phenylnicotinonitrile (10a) Colorless solid (dioxane/ethanol, 88%); m.p. 194–196 °C; IR (υ cm^{-1}): 3437 (OH), 2233 (CN), 1675 (CO); ^{1}H-NMR (DMSO-d_6): δ 3.85 (s, 3H, OCH_3), 4.66 (s, 2H, SCH_2), 6.95 (d, 1H, Ar–H), 7.13 (d, 2H, Ar–H's), 7.53 (t, 1H, Ar–H), 7.57 (t, 2H, Ar–H's), 7.63 (d, 1H, Ar–H), 7.73 (d, 2H, Ar–H's), 7.78 (s, 1H, Ar–H), 7.81 (s, 1H, pyridine-H5), 8.27 (d, 2H, Ar–H's), 10.20 (s, 1H, CHO), 10.68 (s, 1H, OH); ^{13}C-NMR (DMSO-d_6): δ 33.4 (S*C*H_2), 55.9 (O*C*H_3), 102.6, 114.7, 115.2, 115.8, 117.9, 122.5, 128.0, 128.5, 128.9, 129.0, 129.0, 130.5, 130.7, 135.1, 137.2, 153.4, 158.1, 160.3, 161.1, 161.8, 191.6 (*C*O); MS (*m*/*z*): 452 (M^+, 74.6%); Anal. for $C_{27}H_{20}N_2O_3S$: C, 71.66; H, 4.45; N, 6.19; found: C, 71.79; H, 4.31; N, 6.04%.

6-(4-Chlorophenyl)-2-((3-formyl-4-hydroxybenzyl)thio)-4-(4-methoxyphenyl)nicotinonitrile (10b) Colorless solid (dioxane/ethanol, 94%); m.p. 130–132 °C; IR (υ cm^{-1}): 3422 (OH), 2233 (CN), 1676 (CO); ^{1}H-NMR (DMSO-d_6): δ 3.83 (s, 3H, OCH_3), 4.65 (s, 2H, SCH_2), 6.95 (d, 1H, Ar–H), 7.10 (d, 2H, Ar–H's), 7.52 (d, 2H, Ar–H's), 7.65 (d, 1H, Ar–H), 7.71 (d, 2H, Ar–H's), 7.79 (s, 1H, Ar–H), 7.82 (s, 1H, pyridine-H5), 8.16 (d, 2H, Ar–H's), 10.21 (s, 1H, CHO), 10.68 (s, 1H, OH); ^{13}C-NMR (DMSO-d_6): δ 33.5 (S*C*H_2), 55.9 (O*C*H_3), 102.8, 114.6, 115.8, 116.4, 117.8, 122.6, 128.1, 128.9, 129.0, 129.1, 130.5, 130.7, 135.0, 135.7, 137.4, 153.7, 158.7, 160.3, 161.5, 162.4, 191.9 (*C*O); Anal. for $C_{27}H_{19}ClN_2O_3S$ (486.9): C, 66.59; H, 3.93; N, 5.75; found: C, 66.44; H, 3.87; N, 5.82%.

2-((3-Formyl-4-hydroxybenzyl)thio)-4-(4-methoxyphenyl)-6-(*p*-tolyl)nicotinonitrile (10c) Colorless solid (dioxane/ethanol, 93%); m.p. 100–102 °C; IR (υ cm^{-1}): 3439 (OH), 2232 (CN), 1677 (CO); δ 2.38 (s, 3H, *p*-CH_3), 3.84 (s, 3H, OCH_3), 4.65 (s, 2H, SCH_2), 6.95 (d, 1H, Ar–H), 7.13 (d, 2H, Ar–H's), 7.34 (d, 2H, Ar–H's), 7.62 (d, 1H, Ar–H), 7.71 (d, 2H, Ar–H's), 7.80 (s, 1H, Ar–H), 7.82 (s, 1H, pyridine-H5), 8.16 (d, 2H, Ar–H's), 10.21 (s, 1H, CHO), 10.68 (s, 1H, OH); ^{13}C-NMR (DMSO-d_6): δ 21.4 (*p*-*C*H_3), 33.4 (S*C*H_2), 55.9 (O*C*H_3), 102.4, 114.7, 116.1, 116.4, 118.0, 122.5, 127.9, 128.7, 128.9, 129.4, 130.5, 130.9, 134.4, 137.3, 141.1, 154.2, 158.3, 160.4, 161.3, 162.4, 191.5 (*C*O); Anal. for $C_{28}H_{22}N_2O_3S$ (466.5): C, 72.08; H, 4.75; N, 6.00; found: C, 72.23; H, 4.61; N, 6.09%.

2-((3-Formyl-4-hydroxybenzyl)thio)-4,6-bis(4-methoxyphenyl)nicotinonitrile (10d) Colorless solid (dioxane/ethanol, 90%); m.p. 120–122 °C; IR (υ cm^{-1}): 3431 (OH), 2228 (CN), 1671 (CO); ^{1}H-NMR (DMSO-d_6): δ 3.83 (s, 6H, 2 OCH_3), 4.63 (s, 2H, SCH_2), 6.95 (d, 1H, Ar–H), 7.05 (d, 2H, Ar–H's), 7.12 (d, 2H, Ar–H's), 7.67 (d, 1H, Ar–H), 7.75–7.79 (m, 4H, 3 Ar–H's and pyridine-H5), 8.22 (d, 2H, Ar–H's), 10.21 (s, 1H, CHO), 10.69 (s, 1H, OH); ^{13}C-NMR (DMSO-d_6): δ 32.8 (S*C*H_2), 55.3 (2 O*C*H_3), 101.2, 114.2, 114.3, 115.0, 116.0, 117.4, 122.0, 127.7, 128.3, 128.9, 129.0, 129.1, 130.1, 136.7, 153.5, 157.5, 159.8, 160.7, 161.4, 161.9, 191.9 (*C*O); MS (*m*/*z*): 482 (M^+, 70.4%); Anal. for $C_{28}H_{22}N_2O_4S$: C, 69.69; H, 4.60; N, 5.81; found: C, 69.77; H, 4.59; N, 5.70%.

2-((3-Formyl-4-hydroxybenzyl)thio)-4-(4-methoxyphenyl)-6-(4-nitrophenyl)nicotinonitrile (10e) Colorless solid (dioxane, 92%); m.p. 128–130 °C; IR (υ cm^{-1}): 3427 (OH), 2232 (CN), 1679 (CO); ^{1}H-NMR (DMSO-d_6): δ 3.84 (s, 3H, OCH_3), 4.64 (s, 2H, SCH_2), 6.95 (d, 1H, Ar–H), 7.08 (d, 2H, Ar–H's), 7.62 (d, 1H, Ar–H), 7.78 (s, 1H, Ar–H), 7.80 (s, 1H, pyridine-H5), 8.05 (d, 2H, Ar–H's), 8.25 (d, 2H, Ar–H's), 8.38 (d, 2H, Ar–H's), 10.22 (s, 1H, CHO), 10.65 (s, 1H, OH); ^{13}C-NMR (DMSO-d_6): δ 33.8 (S*C*H_2), 55.6 (O*C*H_3), 104.4, 114.8, 115.6, 116.3, 117.9, 122.4, 122.9, 124.5, 128.8, 129.1, 130.5, 130.7, 137.1, 138.7, 144.7, 153.5, 157.5, 160.2, 161.0, 162.3, 191.5 (*C*O); Anal. for $C_{27}H_{19}N_3O_5S$ (497.5): C, 65.18; H, 3.85; N, 8.45; found: C, 65.18; H, 3.85; N, 8.45%.

General procedure for the synthesis of 2-(((3-acetyl-2-oxo-2*H*-chromen-6-yl)methyl)thio)nicotinonitrile derivatives 12(13) A mixture of 2-hydroxybenzaldehydes **9(10)** (5 mmol) and ethyl acetoacetate **11** (5 mmol) in ethanol (15 mL) in the presence of piperazine citrate (10 mol%) was heated at 80 °C for 3 h. The reaction mixture was cooled, filtrated, washed with each of water and cold ethanol, and the reaction product was recrystallized from the proper solvent.

2-(((3-Acetyl-2-oxo-2*H*-chromen-6-yl)methyl)thio)-4,6-diphenylnicotinonitrile (12a) Yellow solid (dioxane, 84%); m.p. 230–233 °C; IR (υ cm^{-1}): 2232 (CN), 1725, 1677 (2 CO); ^{1}H-NMR (DMSO-d_6): δ 2.55 (s, 3H, $COCH_3$), 4.77 (s, 2H, SCH_2), 7.37 (d, 1H, chromene-H8), 7.48 (t, 1H, Ar–H), 7.52 (t, 2H, Ar–H's), 7.56 (t, 1H, Ar–H), 7.60 (t, 2H, Ar–H's), 7.80 (d, 1H, chromene-H7), 7.83 (s, 1H, pyridine-H5), 7.94 (d, 2H, Ar–H's), 8.02 (s, 1H, chromene-H5), 8.24 (d, 2H, Ar–H's), 8.50 (s, 1H,

chromene-H4); Anal. for $C_{30}H_{20}N_2O_3S$ (488.5): C, 73.75; H, 4.13; N, 5.73; found: C, 73.92; H, 4.24; N, 5.55%.

2-(((3-Acetyl-2-oxo-2*H*-chromen-6-yl)methyl) thio)-6-(4-chlorophenyl)-4-phenylnicotinonitrile (12b) Yellow solid (dioxane, 88%); m.p. 236–238 °C; IR (υ cm^{-1}): 2232 (CN), 1723, 1676 (2 CO); ^{1}H-NMR (DMSO-d_6): δ 2.55 (s, 3H, $COCH_3$), 4.69 (s, 2H, SCH_2), 7.38 (d, 1H, chromene-H8), 7.47 (d, 2H, Ar–H's), 7.57 (t, 1H, Ar–H), 7.61 (t, 2H, Ar–H's), 7.81 (d, 1H, chromene-H7), 7.84 (s, 1H, pyridine-H5), 7.94 (d, 2H, Ar–H's), 8.01 (s, 1H, chromene-H5), 8.10 (d, 2H, Ar–H's), 8.52 (s, 1H, chromene-H4); ^{13}C-NMR (DMSO-d_6): δ 30.6 (CH_3), 33.5 (SCH_2), 103.9, 116.1, 116.5, 116.9, 118.2, 125.2, 127.9, 128.2, 128.3, 129.0, 129.6, 130.5, 135.2, 135.3, 135.5, 135.8, 136.4, 146.9 153.9, 157.9, 158.7, 161.7, 162.3 (chromene-*C*O), 195.2 (*C*O); Anal. for $C_{30}H_{19}ClN_2O_3S$ (523.0): C, 68.90; H, 3.66; N, 5.36; found: C, 68.74; H, 3.85; N, 5.47%.

2-(((3-Acetyl-2-oxo-2*H*-chromen-6-yl)methyl) thio)-4-phenyl-6-(*p*-tolyl)nicotinonitrile (12c) Yellow solid (dioxane, 86%); m.p. 216–219 °C; IR (υ cm^{-1}): 2232 (CN), 1722, 1678 (2 CO); ^{1}H-NMR (DMSO-d_6): δ 2.39 (s, 3H, *p*-CH_3), 2.56 (s, 3H, $COCH_3$), 4.78 (s, 2H, SCH_2), 7.34 (d, 2H, Ar–H's), 7.40 (d, 1H, chromene-H8), 7.55 (t, 1H, Ar–H), 7.59 (t, 2H, Ar–H's), 7.82 (d, 1H, chromene-H7), 7.85 (s, 1H, pyridine-H5), 7.94 (d, 2H, Ar–H's), 8.05 (s, 1H, chromene-H5), 8.13 (d, 2H, Ar–H's), 8.52 (s, 1H, chromene-H4); Anal. for $C_{31}H_{22}N_2O_3S$ (502.5): C, 74.08; H, 4.41; N, 5.57; found: C, 73.85; H, 4.57; N, 5.40%.

2-(((3-Acetyl-2-oxo-2*H*-chromen-6-yl)methyl) thio)-6-(4-methoxyphenyl)-4-phenylnicotinonitrile (12d) Yellow solid (dioxane/ethanol, 84%); m.p. 220–222 °C; IR (υ cm^{-1}): 2233 (CN), 1720, 1676 (2 CO); ^{1}H-NMR (DMSO-d_6): δ 2.55 (s, 3H, $COCH_3$), 3.85 (s, 3H, OCH_3), 4.76 (s, 2H, SCH_2), 7.08 (d, 2H, Ar–H's), 7.40 (d, 1H, chromene-H8), 7.52 (t, 1H, Ar–H), 7.57 (t, 2H, Ar–H's), 7.84 (d, 1H, chromene-H7), 7.87 (s, 1H, pyridine-H5), 7.95 (d, 2H, Ar–H's), 8.04 (s, 1H, chromene-H5), 8.24 (d, 2H, Ar–H's), 8.50 (s, 1H, chromene-H4); ^{13}C-NMR (DMSO-d_6): δ 30.4 (CH_3), 33.4 (SCH_2), 55.3 (OCH_3), 102.4, 114.3, 116.3, 116.8, 116.9, 118.2, 124.9, 127.8, 128.3, 128.9, 129.5, 130.3, 130.4, 135.1, 135.4, 136.1, 146.6, 154.6, 157.8, 158.1, 158.3, 162.0, 162.3 (chromene-*C*O), 195.1 (*C*O); MS (*m*/*z*): 518 (M^+, 54.2%); Anal. for $C_{31}H_{22}N_2O_4S$: C, 71.80; H, 4.28; N, 5.40; found: C, 71.60; H, 4.37; N, 5.53%.

2-(((3-Acetyl-2-oxo-2*H*-chromen-6-yl)methyl) thio)-6-(4-nitrophenyl)-4-phenylnicotinonitrile (12e) Yellow solid (dioxane, 90%); m.p. 244–247 °C; IR (υ cm^{-1}): 2231 (CN), 1724, 1674 (2 CO); ^{1}H-NMR (DMSO-d_6): δ 2.55 (s, 3H, $COCH_3$), 4.76 (s, 2H, SCH_2), 7.39 (d, 1H, chromene-H8), 7.53 (t, 1H, Ar–H), 7.58 (t, 2H, Ar–H's), 7.79 (d, 1H, chromene-H7), 7.82 (s, 1H, pyridine-H5), 7.96 (d, 2H, Ar–H's), 8.03 (s, 1H, chromene-H5), 8.22 (d, 2H, Ar–H's), 8.41 (d, 2H, Ar–H's), 8.52 (s, 1H, chromene-H4); Anal. for $C_{30}H_{19}N_3O_5S$ (533.5): C, 67.53; H, 3.59; N, 7.88; found: C, 67.41; H, 3.72; N, 7.64%.

2-(((3-Acetyl-2-oxo-2*H*-chromen-6-yl)methyl) thio)-4-(4-methoxyphenyl)-6-phenylnicotinonitrile (13a) Yellow solid (dioxane, 89%); m.p. 226–228 °C; IR (υ cm^{-1}): 2231 (CN), 1721, 1675 (2 CO); ^{1}H-NMR (DMSO-d_6): δ 2.55 (s, 3H, $COCH_3$), 3.84 (s, 3H, OCH_3), 4.78 (s, 2H, SCH_2), 7.13 (d, 2H, Ar–H's), 7.42 (d, 1H, chromene-H8), 7.53 (t, 1H, Ar–H), 7.57 (t, 2H, Ar–H's), 7.73 (d, 2H, Ar–H's), 7.84 (d, 1H, chromene-H7), 7.87 (s, 1H, pyridine-H5), 8.05 (s, 1H, chromene-H5), 8.24 (d, 2H, Ar–H's), 8.50 (s, 1H, chromene-H4); ^{13}C-NMR (DMSO-d_6): δ 30.5 (CH_3), 33.5 (SCH_2), 55.9 (OCH_3), 102.4, 114.5, 115.3, 115.6, 116.3, 117.9, 125.1, 128.0, 128.5, 128.9, 129.7, 130.6, 130.8, 135.2, 135.3, 135.5, 146.8, 153.4, 158.1, 158.4, 160.2, 161.8, 162.2, 195.1 (*C*O); MS (*m*/*z*): 518 (M^+, 53.0%); Anal. for $C_{31}H_{22}N_2O_4S$: C, 71.80; H, 4.28; N, 5.40; found: C, 71.89; H, 4.51; N, 5.27%.

2-(((3-Acetyl-2-oxo-2*H*-chromen-6-yl)methyl)thio)-6-(4-chlorophenyl)-4-(4-methoxyphenyl)nicotinonitrile (13b) Yellow solid (dioxane, 87%); m.p. 244–246 °C; IR (υ cm^{-1}): 2232 (CN), 1722, 1675 (2 CO); ^{1}H-NMR (DMSO-d_6): δ 2.57 (s, 3H, CH_3), 3.83 (s, 3H, OCH_3), 4.69 (s, 2H, SCH_2), 7.10 (d, 2H, Ar–H's), 7.41 (d, 1H, chromene-H8), 7.52 (d, 2H, Ar–H's), 7.81 (d, 1H, chromene-H7), 7.84 (s, 1H, pyridine-H5), 8.05 (s, 1H, chromene-H5), 8.13 (d, 2H, Ar–H's), 8.24 (d, 2H, Ar–H's), 8.52 (s, 1H, chromene-H4); ^{13}C-NMR (DMSO-d_6): δ 30.4 (CH_3), 33.5 (SCH_2), 55.9 (OCH_3), 103.2, 114.7, 116.3, 116.5, 117.1, 118.5, 125.0, 128.1, 128.8, 129.2, 130.5, 130.7, 135.1, 135.5, 135.6, 135.8, 147.1, 154.0, 158.0, 158.9, 160.3, 161.6, 162.6 (chromene-*C*O), 195.2 (*C*O); Anal. for $C_{31}H_{21}ClN_2O_4S$ (553.0): C, 67.33; H, 3.83; N, 5.07; found: C, 67.09; H, 3.96; N, 5.23%.

2-(((3-Acetyl-2-oxo-2*H*-chromen-6-yl)methyl)thio)-4-(4-methoxyphenyl)-6-(*p*-tolyl)nicotinonitrile (13c) Yellow solid (dioxane/ethanol, 85%); m.p. 218–220 °C; IR (υ cm^{-1}): 2230 (CN), 1723, 1675 (2 CO); ^{1}H-NMR (DMSO-d_6): δ 2.39 (s, 3H, *p*-CH_3), 2.55 (s, 3H, $COCH_3$), 3.84 (s, 3H, OCH_3), 4.77 (s, 2H, SCH_2), 7.12 (d, 2H, Ar–H's), 7.34 (d, 2H, Ar–H's), 7.41 (d, 1H, chromene-H8), 7.71 (d, 2H, Ar–H's), 7.82 (d, 1H, chromene-H7), 7.87 (s, 1H, pyridine-H5), 8.04 (s, 1H, chromene-H5), 8.13 (d, 2H, Ar–H's), 8.49 (s, 1H, chromene-H4); ^{13}C-NMR (DMSO-d_6): δ 21.3 (*p*-CH_3), 30.6 (CH_3), 33.4 (SCH_2), 55.9 (OCH_3), 102.6, 114.5, 116.0, 116.5, 116.7, 118.1, 125.3, 127.8, 128.8, 128.9, 130.5,

130.7, 134.4, 135.0, 135.2, 141.3, 146.6, 154.3, 157.5, 158.0, 160.2, 161.7, 162.1 (chromene-*C*O), 195.0 (*C*O); MS (*m*/*z*): 532 (M^+, 66.2%); Anal. for $C_{32}H_{24}N_2O_4S$: C, 72.16; H, 4.54; N, 5.26; found: C, 72.02; H, 4.66; N, 5.32%.

2-(((3-Acetyl-2-oxo-2*H*-chromen-6-yl)methyl) thio)-4,6-bis(4-methoxyphenyl)nicotinonitrile (13d) Yellow solid (dioxane, 89%); m.p. 182–184 °C; IR (υ cm^{-1}): 2231 (CN), 1725, 1674 (2 CO); ^{1}H-NMR (DMSO-d_6): δ 2.53 (s, 3H, $COCH_3$), 3.81 (s, 6H, 2 OCH_3), 4.69 (s, 2H, SCH_2), 7.01 (d, 2H, Ar–H's), 7.11 (d, 2H, Ar–H's), 7.36 (d, 1H, chromene-H8), 7.65 (d, 2H, Ar–H's), 7.71 (s, 1H, pyridine-H5), 7.80 (d, 1H, chromene-H7), 7.98 (s, 1H, chromene-H5), 8.16 (d, 2H, Ar–H's), 8.46 (s, 1H, chromene-H4); ^{13}C-NMR (DMSO-d_6): δ 29.9 (CH_3), 32.8 (SCH_2), 55.3 (2 OCH_3), 101.2, 114.2, 114.3, 115.1, 115.9, 116.2, 117.8, 124.4, 127.7, 128.9, 129.1, 130.1, 130.4, 134.3, 134.8, 146.5, 153.5, 153.6, 157.5, 158.2, 160.5, 161.4, 161.6 (chromene-*C*O) 194.9 (*C*O); Anal. for $C_{32}H_{24}N_2O_5S$ (548.6): C, 70.06; H, 4.41; N, 5.11; found: C, 70.23; H, 4.48; N, 4.99%.

2-(((3-Acetyl-2-oxo-2*H*-chromen-6-yl)methyl)thio)-4-(4-methoxyphenyl)-6-(4-nitrophenyl)nicotinonitrile (13e) Yellow solid (dioxane, 86%); m.p. 240–241 °C; IR (υ cm^{-1}): 2232 (CN), 1725, 1676 (2 CO); ^{1}H-NMR (DMSO-d_6): δ 2.55 (s, 3H, $COCH_3$), 3.83 (s, 3H, OCH_3), 4.77 (s, 2H, SCH_2), 7.11 (d, 2H, Ar–H's), 7.39 (d, 1H, chromene-H8), 7.79 (d, 1H, chromene-H7), 7.83 (s, 1H, pyridine-H5), 8.05 (s, 1H, chromene-H5), 8.22–8.25 (m, 4H, Ar–H's), 8.40 (d, 2H, Ar–H's), 8.51 (s, 1H, chromene-H4); Anal. for $C_{31}H_{21}N_3O_6S$ (563.5): C, 66.07; H, 3.76; N, 7.46; found: C, 65.93; H, 3.86; N, 7.32%.

AChE inhibition assay

Assessment of the AChE inhibitory activity was carried out using the previously stated Ellman method [47, 54] with slight modifications. In a mixture of DMSO and methanol (1 and 9 mL, respectively), the stock solutions of the tested compounds were prepared, followed by dilution in the buffer KH_2PO4/K_2HPO4 (50 mM, pH 7.7) to obtain final test concentrations. Ten microliters of the respective assayed sample (at stock solution of 0.5 mM) was added to 60 µL of phosphate buffer (50 mM, pH 7.7). Then, 10 µL of 0.005 unit per well enzyme solution (AChE, E.C. 3.1.1.7, Type V-S, lyophilized powder, from *electric eel*, 1000 units, Sigma-Aldrich) was added. The resulting content was mixed and pre-read at 405 nm and then incubated at 37° C for 10 min. In each well, the reaction was started by adding 10 µL of acetylthiocholine iodide (0.5 mM), followed by adding 10 µL of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB, 0.5 mM per well). At 37 °C, the wells were incubated, and then, the absorption of each well was measured at 405 nm using the 96-well plate reader Synergy HT, Biotek, USA. Eserine (0.5 mM) was used as a positive control. The inhibition percentages were calculated by the following formula:

$$\text{Inhibition}\% = \left(\text{Control}_{\text{absorbance}} - \text{Sample}_{\text{absorbance}}\right) \times 100/\text{Control}_{\text{absorbance}}$$

Five different concentrations of each compound were tested in triplicate. The IC_{50} values were determined using sigma plot software.

DPPH radical-scavenging assay

DPPH radical-scavenging activity was measured according to Thuong et al. method [55] with slight modifications. The new hybrids have been examined within the range of 0–25 µg/mL using methanol as a solvent. In 96-well plates, 1 mL of methanolic DPPH solution (0.3 mM) was added to 2.5 mL of tested derivative in 5 different concentrations. One milliliter of methanol was added, and the solution was mixed for a minute and incubated in a dark place at room temperature. After thirty minutes, the absorbance of the reaction mixture was measured at 520 nm on a microplate reader. The reading blank consisted of 2.5 mL of tested derivative and 1 mL of methanol; meanwhile, the mixture of 1 mL DPPH and 2.5 mL of methanol was used as negative control. The percent of the antioxidant activity was calculated using the equation:

$$\text{Inhibition}\% = \left(\text{Blank sample}_{\text{absorbance}} - \text{Sample}_{\text{absorbance}}\right) \times 100/\text{Blank sample}_{\text{absorbance}}$$

The IC_{50} value was calculated and compared with ascorbic acid as a standard drug.

Cytotoxicity

Cell line, culture conditions and preparation of compounds

The human breast epithelial cell line (MCF-10A), human breast carcinoma cell line (MCF-7), colon cancer cell line (Caco2) and liver hepatocellular carcinoma cell line (HEPG2) were obtained from ATCC via Holding company for biological products and vaccines (VACSERA), Cairo, Egypt [56].

The selected cell lines were cultivated in Dulbecco's Modified Eagle's Medium (DMEM). All of the growth media were supplemented with 10% Fetal Bovine Serum (FBS) and antibiotics (100 U/mL penicillin and 100 mg/mL streptomycin) at 37 °C in a humidified atmosphere containing 5% CO_2. New hybrid derivatives **12b**, **12e**, **13b** and **13e** as well as Doxorubicin, as a positive control, were dissolved in DMSO, and final concentrations were diluted in culture medium.

Neutral red uptake assay (NRU assay)

The NRU assay depends on the ability of living cells to bind neutral red, in lysosomes [57]. The cytotoxicity of new hybrid derivatives **12b**, **12e**, **13b** and **13e** were evaluated against each of MCF-10A, MCF-7, Caco2 and HEPG2 cell lines using Doxorubicin as a standard drug. Exponentially growing cells were collected using 0.25% Trypsin–EDTA; then, the cell suspension was counted using hemocytometer, and cell viability was checked by trypan blue (100% viability). Then, the cells suspension was diluted with complete medium to have an approximately 1.0×10^5 cell/mL; then, 200 µL of the cell suspension was dispensed by multichannel pipette into the inner 60 wells of the 96-well plate, and the peripheral wells were filled with PBS, and then, the plate was incubated for 24 h before treatment with the tested compounds to allow attachment of cells to the wall of the plate. Different concentrations of the tested derivatives (5, 25, 50 and 75 µg/mL) were prepared using DMEM media. 200 µL of treatment media was dispensed into 4 replicates for each concentration, other wells were filled with untreated cells only (as a negative control) and wells filled with media containing Doxorubicin HCL as a positive control. The 96-well plate was incubated at 37° C for 48 h. Then, the medium and extracts were discarded and replaced with 100 µL of neutral red solution (50 mg/mL) and centrifuged at 1800 rpm for ten min to remove any crystals of dye. After incubation at 37 °C for 3 h, the dye medium was discarded and the microplate was washed twice with 150 µL PBS to remove the unabsorbed neutral red dye contained in the wells. The cellular morphology of each of the treated MCF-10A, MCF-7, Caco2 and HEPG2 cell lines with the tested derivatives was observed using Inverted Microscope Leica DMI3000B. The absorbance of acidified ethanol solution containing extracted neutral red dye was determined using microplate reader (BioTek, ELX808) at 540 nm to estimate the optical density, and the cell viability percentage was measured.

The molecular docking: a tool of in silico studies

The in silico research was performed by study of molecular docking with AChE (PDB ID: 4EY7) for some new coumarins. In this study, a rigid framework for the Molecular Operating Environment (MOE) version 2019.01 was used [58]. MOE is one of the simple interactive molecular graphic tools used to test the feasible modes of docking of the set of ligands with COX-2 enzyme. The input of both the tested ligands and AChE must be in PDB format. The Gaussian 03 software was used to create a PDB file format of the structure of each ligand. The AChE structure was obtained from the web site of protein data bank (https:/www.rcsb.org/). Only the amino acid chain is maintained, while other molecules such as co-crystallized ligands, water and other unsupported elements (e.g., Na, Mg, etc.) are detached [59, 60].

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