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Synthesis and in vitro Study of new Coumarin Derivatives Linked to Nicotinonitrile Moieties as Potential Acetylcholinesterase Inhibitors

Ahmed E. M. Mekky and Sherif M. H. Sanad*

Chemistry Department, Faculty of Science, Cairo University, Giza 12613, Egypt

E-mail: sherif_hamed1980@yahoo.com

Abstract

The appropriate pyridine-2(1*H*)-thiones were reacted with an equivalent amount of 5-(chloromethyl)-2-hydroxybenzaldehyde in ethanol in the presence of potassium hydroxide to give the corresponding 2-hydroxybenzaldehyde derivatives in excellent yields. The latter derivatives were taken as key synthons for the preparation of the target hybrids. Therefore, 2-hydroxybenzaldehydes were reacted with benzoylglycine in acetic anhydride in the presence of fused sodium acetate at 100 °C for 6 h to afford a new series of nicotinonitrile-coumarin hybrids. The in vitro acetylcholinesterase inhibitory activities were estimated for the new coumarins. The results were expressed as the inhibition percentage of the tested hybrids at concentration of 25 nM, compared to donepezil as a reference (inhibition percentage of 70.5). Coumarin hybrids linked to 6-(4-nitrophenyl) or 6-(4-chlorophenyl)-4-phenylnicotinonitrile exhibited more effective inhibitory activities than donepezil with inhibition percentages of 94.1 and 72.3, respectively. The new coumarins were tested for their free radical-scavenging capabilities against DPPH. Furthermore, some new coumarins were tested for in vitro cytotoxic activity against each MCF-10A, MCF-7, Caco2, and HEPG2. The new hybrids showed cytotoxicity in micromolar range (IC₅₀ of 3.5-13.9 μM) against all tested cell lines. These results clearly demonstrated that the hybrids being tested are not cytotoxic at the concentration required to inhibit acetylcholinesterase effectively.

Keywords: Alzheimer's disease, nicotinonitrile-coumarin hybrids, acetylcholinesterase inhibitors, antioxidant activity, in vitro cytotoxicity screening.

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1. Introduction

Alzheimer's disease (AD) is a progressive and degenerative brain condition that mainly affects the elderly and contributes to memory loss and everyday activity capacity. AD is the main cause of dementia that affects around 50 million people around the world, with nearly 10 million new patients per year, accounting for 60-70 per cent of dementia cases.^[1-3]

The real origin of AD remains an open question, however, and thus one of the exciting areas of current research in medicinal chemistry is the development of an effective treatment of AD.^[4] During the last 2 decades, several hippocampal anatomy studies, which relate to learning and perception, have shown a substantial decrease in the rates of neurotransmitter acetylcholine in AD patients.^[5-7] Nowadays, clinical research has centered on the issue of loss of basal forebrain cholinergic activity, as it is the only evidence responsible for cognitive impairment and neurodegeneration in AD patients' brains.^[8-10]

Many strategies have been introduced to improve cholinergic neurotransmission such as improved acetylcholine level (ACh) synthesis or pre-synaptic release and stimulation of post-synaptic muscarinic and nicotinic receptors, and to reduce ACh synaptic degradation by inhibitors of acetylcholinesterase (AChE).^[11-18] Donepezil, tacrine, galantamine, and rivastigmine are currently approved AChE inhibitors (see Figure 1).^[19]

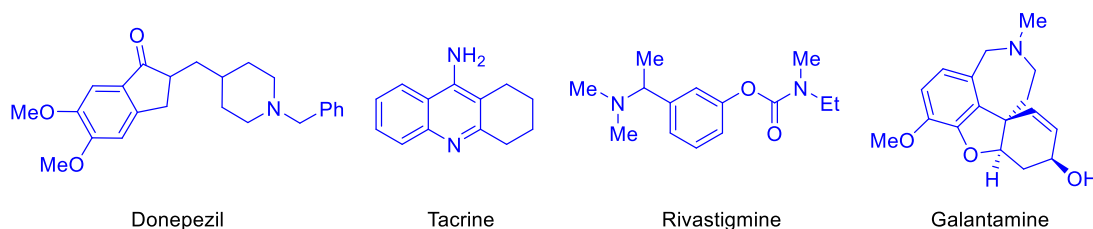


Figure 1. Structure of some inhibitors of AChE.

Coumarin derivatives are natural heterocycles which are included in many diseases' inhibition and cure. Derivatives bearing coumarin moiety act as antimicrobials,^[20] anti-inflammatory agents,^[21] anticancer agents,^[22] anti-Alzheimer's disease,^[23,24] and anti-Parkinson agents.^[25] Dicoumarol and warfarin are examples of chromenes considered effective oral anticoagulants.^[26] Furthermore, the moiety of coumarin was used in several successful inhibitors of AChE.^[27-30]

In the current study, we report the preparation of a new series of nicotinonitrile-coumarin hybrids, incorporating arene groups. These hybrids have been subjected to in vitro study as possible inhibitors of AChE.

2. Results and discussion

2.1. Chemistry

AChE is an enzyme present in cholinergic neurons and its key role is the rapid breakdown of the neurotransmitter ACh released throughout neurotransmission.^[31] The active site of the enzyme is a 20 Å length gorge that contains a catalytic anionic site (CAS) and a peripheral anionic binding site (PAS). Both sites play a central role in the enzyme catalysis,^[32] where CAS is responsible for the correct orientation and stabilization of the trimethylammonium group of ACh. In addition, PAS promotes allosteric control at the entrance to the cavity. Donepezil had been reported to bind to both PAS and CAS [32] simultaneously, where indanone moiety forms several H-bonding and π - π interactions with PAS aminoacid residues. In addition, its piperidine moiety forms multiple π - π stacking interactions with CAS aminoacid residues.^[33-35]

Coumarin group is typically integrated in PAS because of its planarity, steric complementarity, and potential π - π stacking, whereas a net positive charge normally allows for strong cation- π interactions in CAS (see Figure 2).^[29, 30]

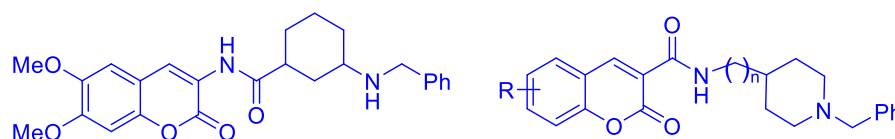


Figure 2. Structure of some potent AChE inhibitors with nanomolar affinities.

We recently reported the promising antibacterial and anticancer activities of novel nicotinonitrile-coumarin hybrids.^[36] Stimulated by these findings as well as the resemblance of these hybrids and donepezil (see Figure 3), we report herein the preparation of new hybrids as potent AChE inhibitors. To promote the interaction of new hybrids with the active gorge, *N*-(2-oxo-2*H*-chromen-3-yl)benzamides linked to nicotinonitriles *via* thioethers were included in their structures. These moieties replace indanone and piperidine moieties in donepezil respectively.

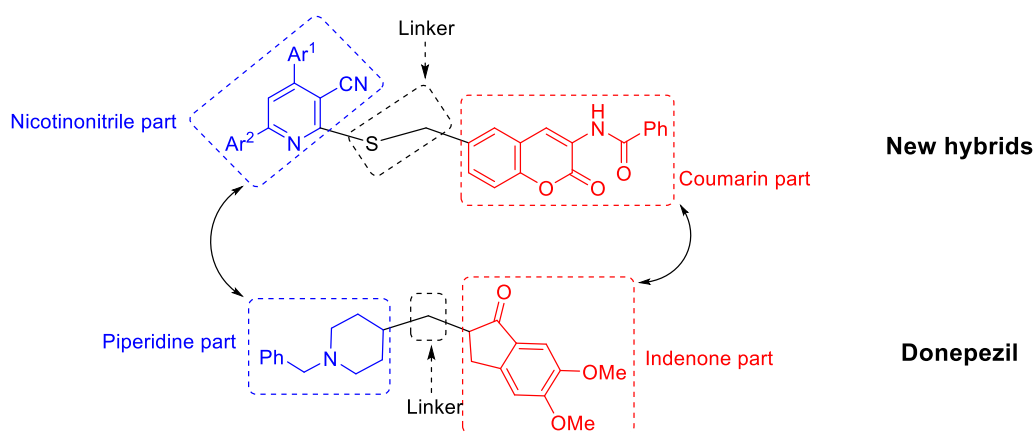
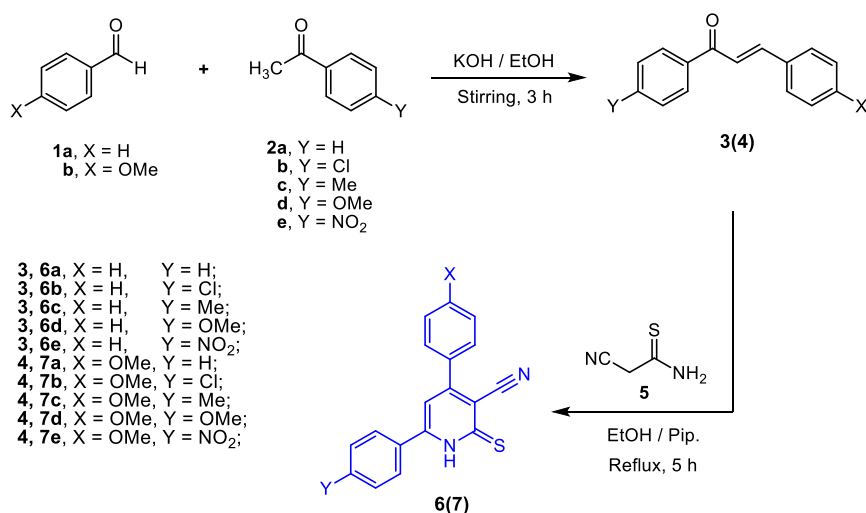


Figure 3. Structure of new hybrids and donepezil.

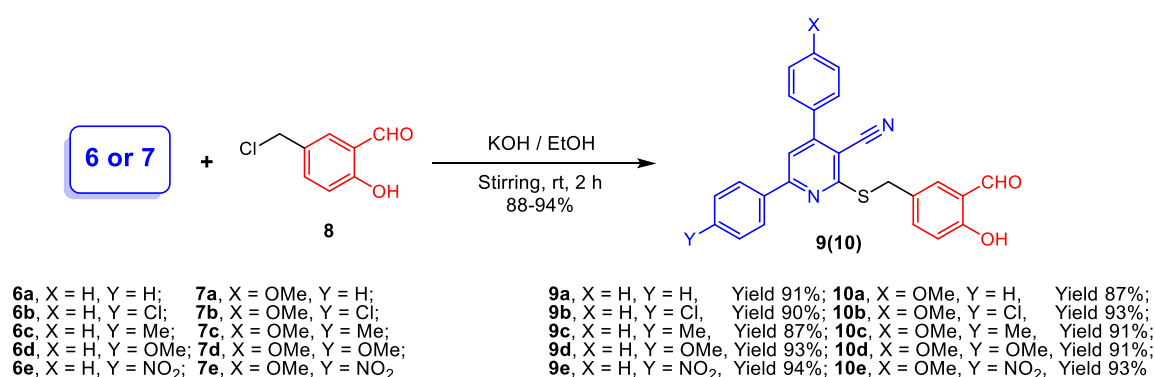
To achieve our target, we prepare initially a diverse series of nicotinonitriles. Therefore, each of acetophenones **2** was reacted with benzaldehyde **1a** or anisaldehyde **1b** in ethanolic potassium hydroxide solution under stirring at room temperature (rt) for 3 h to afford a series of α,β -unsaturated carbonyl derivatives **3(4)**.^[37-41] Each of the latter derivatives were cyclocondensed with 2-cyanothioacetamide **5** in ethanol in the presence of piperidine at reflux for 5 h afforded the key intermediates 2-thioxopyridine-3-carbonitriles **6(7)**^[42-44] (Scheme 1).



Scheme 1. Preparation of 2-thioxopyridine-3-carbonitriles **6(7)**.

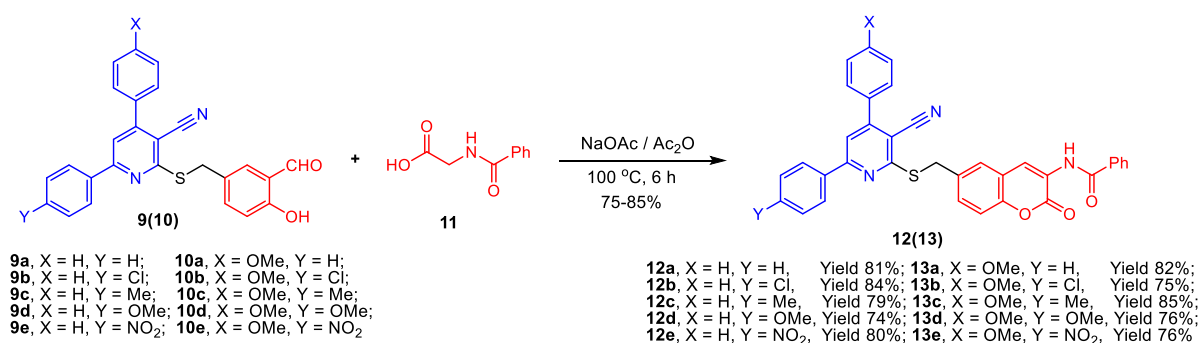
The preparation of a new series of 2-hydroxybenzaldehyde-nicotinonitrile derivatives **9(10)**, in excellent yields, was our next step. Therefore, each of 2-thioxopyridine-3-carbonitriles **6(7)** was stirred in ethanolic potassium hydroxide solution at rt for 10 minutes. To the mixture, 5-(chloromethyl)-2-hydroxybenzaldehyde **8**^[45] was added. For further 2 h stirring, the reaction was continued to afford the 2-hydroxybenzaldehyde-nicotinonitriles **9a-9e** and **10a-10e** in 88-94% yields (Scheme 2). The IR spectrum of **9d**, as a representative

example, showed three bands at 3414, 2231 and 1674 cm^{-1} corresponding to OH, CN and CO groups, respectively. A molecular ion peak at $m/z = 452$ appeared in its mass spectrum. Its ^1H -NMR spectrum showed four singlet signals at δ 3.83, 4.64, 10.22 and 10.63 attributed to protons of CH_3 , CH_2 , CHO and OH, respectively. Its ^{13}C -NMR spectrum revealed three signals at δ 33.5, 55.3 and 191.7 assigned to carbons of CH_2 , CH_3 and CO, respectively (see experimental section).



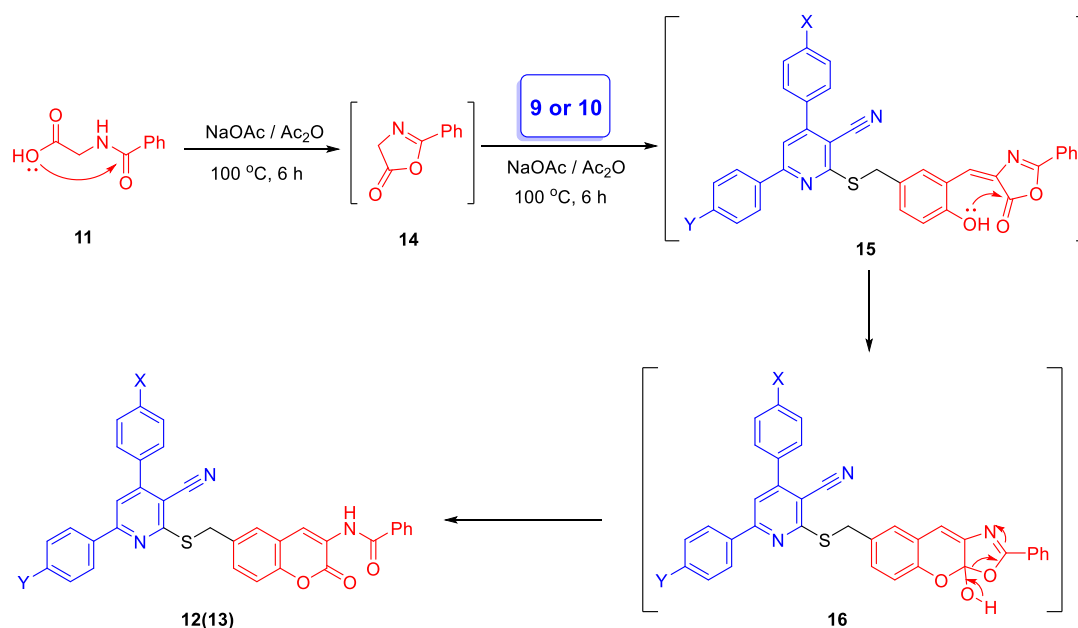
Scheme 2. Preparation of 2-hydroxybenzaldehyde-nicotinonitrile derivatives **9(10)**.

Subsequently, 2-hydroxybenzaldehyde-nicotinonitriles **9(10)** were taken as intermediates for the preparation of the target coumarin hybrids **12(13)**. Thus, compounds **9(10)** were reacted with benzoylglycine **11**. The reaction was carried out in acetic anhydride containing a catalytic amount of fused sodium acetate. The mixture was heated at 100 °C for 6 h to afford *N*-(6-(((3-cyanopyridin-2-yl)thio)methyl)-2-oxo-2*H*-chromen-3-yl)benzamides **12(13)** in 74-85% yields (Scheme 3).^[46] The IR spectrum of **13c**, as a typical example, showed NH, CN and two CO bands at 3336, 2230, 1687 and 1641 cm^{-1} , respectively. At $m/z = 609$, a molecular ion peak appeared in its mass spectrum. The ^1H -NMR spectrum of **13c** revealed four singlet signals at δ 2.36, 3.84, 4.69 and 9.62 corresponding to protons of CH_3 , OCH_3 , CH_2 and NH, respectively. Moreover, it revealed two doublets as well as two singlet signals at δ 7.38, 7.82, 8.02 and 8.33, respectively, corresponding to protons of chromene. Its ^{13}C -NMR spectrum showed five signals at δ 21.5, 33.8, 55.7, 160.5 and 165.3 assigned for carbons of CH_3 , CH_2 , OCH_3 , chromene-CO and CO, respectively (see experimental section).



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

The above reaction may proceed through the initial intramolecular cyclization of benzoylglycine **11** to give **[14]**, followed by its *in-situ* Knoevenagel condensation to give **[15]**. The above intermediate was subjected to the subsequent nucleophilic addition to form intermediate **[16]**, followed by ring opening to give **12(13)** (Scheme 4).^[47]



Scheme 4. The proposed mechanism for the synthesis of coumarins **12(13)**.

2.2. Biology

2.2.1. The *in vitro* AChE inhibitory activity

The new coumarins **12(13)** were tested for their AChE inhibitory activity using Ellman method.^[48] The inhibition percentage of the tested hybrids **12(13)** against AChE at concentration of 25 nM were determined. At this concentration, the reference donepezil gave inhibition percentage of 70.5%.^[49] The results are recorded in Figure 4.

With regards to coumarins **12a-12e**, linked to 6-aryl-4-phenylnicotinonitrile, hybrids **12b** and **12e** displayed the best powerful AChE inhibitory activity. Thus, **12e**, linked to 6-(4-

nitrophenyl) group, exhibited more effective activity than donepezil with inhibition percentage of 94.1. The second in AChE inhibitory strength was **12b**, bearing 6-(4-chlorophenyl) moiety with inhibition percentage of 72.3. Other hybrids **12a**, **12c**, and **12d** showed reduced efficacies. **12a**, bearing 6-phenyl moiety, exhibited inhibition percentage of 14.6. Furthermore, hybrids **12c** and **12d**, bearing 6-(*p*-tolyl) and 6-(4-methoxyphenyl) moiety respectively, exhibited the weakest efficacies with inhibition percentages of 10.7 and 7.4, respectively.

With regards to coumarins **13a-13e**, linked to 6-aryl-4-(4-methoxyphenyl)nicotinonitrile moiety, this series showed in general less efficacies against AChE. Moreover, the inhibitory efficacies of the hybrids examined was in the same order as in coumarins **12**. Therefore, **13b** and **13e**, with 6-(4-chlorophenyl) and 6-(4-nitrophenyl) moiety respectively, were the most effective hybrids with inhibition percentages of 23.9 and 36.6, respectively. Other hybrids **13a**, **13c**, and **13d**, bearing 6-phenyl, 6-(*p*-tolyl) and 6-(4-methoxyphenyl) moiety respectively, showed the weakest efficacies (inhibition percentages of 2.8-5.7).

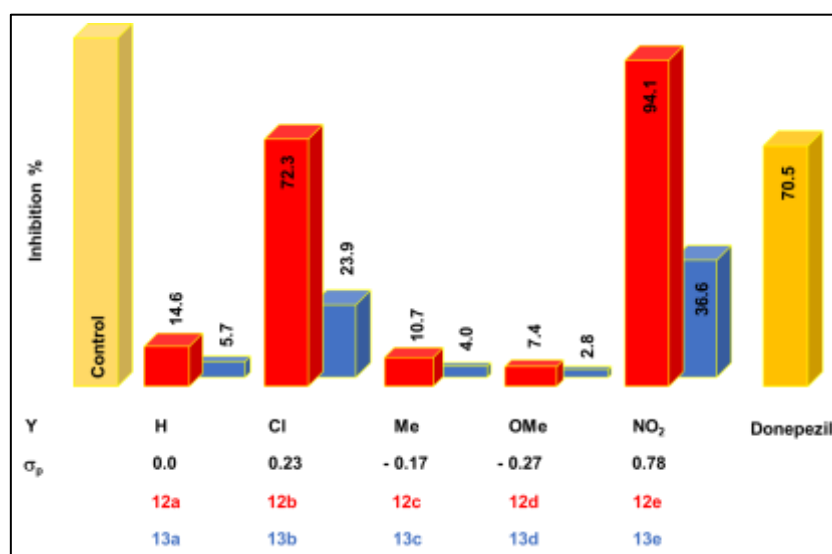


Figure 4. The relation between σ_p and inhibition percentages of the new nicotinonitrile-coumarin hybrids **12(13)** at concentrations of 25 nM. Inhibition percentages were calculated taking negative control as 100% inhibition.

In order to explain the relationship between the electronic properties of the tested coumarins and their AChE inhibitory efficacies, we compare the values of electronic substituent constant (σ_p)^[50] with the inhibition percentages of the new hybrids (Figure 4). The examination of Figure 4 showed an obvious relation between the inhibition percentages

and the values of σ_p . In each series, we found that the efficacies of AChE inhibition improved as the substituent, linked to 6-arylnicotinonitrile, became more electron withdrawal.

2.2.2. DPPH antioxidant activity

Many efforts have been done to explore the success of antioxidant therapeutic strategies in the treatment of neurodegenerative diseases as AD.^[51-53] A subset of amyloid plaques has recently been reported to develop free radicals in living, Alzheimer's models and human Alzheimer's tissues. Such extremely reactive molecules are neutralized by antioxidant therapy and can therefore be of therapeutic benefit in AD.^[54] Depending on the above, the assessment of the antioxidant efficacies of the tested hybrids is considered to provide value for AD diagnostics.

Hence, the free radical quenching capabilities of the tested hybrids were studied against DPPH. The results are expressed as the inhibition percentage of the tested hybrids **12(13)** at concentration of 25 $\mu\text{g/mL}$ (Figure 5). The reference ascorbic acid gave inhibition percentage of 89.8.

Compound **12e** displayed the best efficacy with inhibition percentage of 90.7. Furthermore, compounds **12b**, **13b** and **13e** showed free radical quenching activities with inhibition percentages of 62.9-71.4. Other tested hybrids exhibited decreased capabilities with inhibition percentages in the range of 4.7-17.6.

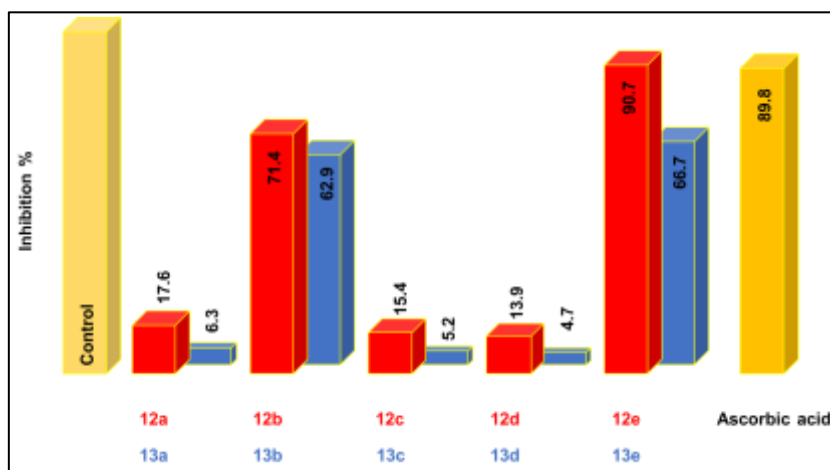


Figure 5. DPPH scavenging activity of the new hybrids at concentrations of 25 $\mu\text{g/mL}$.

Inhibition percentages were calculated taking negative control as 100% inhibition.

2.2.3. Cytotoxicity against eukaryotic cells

The in vitro cytotoxic activities of some hybrids were assessed. For this purpose, each of MCF-10A, MCF-7, Caco2 and HEPG2 cell lines were selected. The findings of IC_{50} (in

μM) are listed in Table 1. The reference Doxorubicin exhibited IC_{50} of 12.2-14.5 μM against the tested cell lines.

Hybrids **12b**, **12e** and **13e** exhibited more effective activities against all tested cell lines when compared with Doxorubicin. **12e** displayed the best efficacies with IC_{50} of 3.5 ± 0.1 , 4.4 ± 0.2 , 4.9 ± 0.2 , and 6.2 ± 0.2 μM against each of the Caco2, HEPG2, MCF-7, and MCF-10A cell lines, respectively. Furthermore, **12b** and **13e** exhibited IC_{50} of 4.9-7.9 μM against the above tested cell lines (Table 1).

With regards to the MCF-7 and Caco2 cell lines, **13b** displayed more powerful efficacies than Doxorubicin with 11.9 ± 0.2 and 12.9 ± 0.3 μM , respectively. Moreover, **13b** showed less powerful efficacies against each of the MCF-10A and HEPG2 cell lines with 13.2 ± 0.3 and 13.9 ± 0.3 μM , respectively, when compared with Doxorubicin (Table 1).

Table 1. The IC_{50} of some new hybrids against each of MCF-10A, MCF-7, Caco2 and HEPG2. Doxorubicin was used as a reference.

Compound	IC_{50} (μM)			
	MCF-10A	MCF-7	Caco2	HEPG2
12b	7.6 ± 0.2	5.6 ± 0.2	5.1 ± 0.1	4.9 ± 0.1
12e	6.2 ± 0.2	4.9 ± 0.2	3.5 ± 0.1	4.4 ± 0.2
13b	13.2 ± 0.3	11.9 ± 0.2	12.9 ± 0.3	13.9 ± 0.3
13e	7.9 ± 0.2	6.2 ± 0.2	5.7 ± 0.2	7.2 ± 0.3
Doxorubicin	12.7 ± 0.3	12.2 ± 0.2	14.5 ± 0.3	13.7 ± 0.3

It is a noteworthy to mention that cytotoxicity of the new hybrids **12b**, **12e**, **13b**, and **13e** showed IC_{50} in micromolar range (IC_{50} of 3.5-13.9 μM) against all tested the cell lines, while some of these tested derivatives showed effective AChE inhibition in nanomolar range (inhibition percentage up to 94.1 at concentration of 25 nM). These findings clearly showed that the new nicotinonitrile-coumarin hybrid derivatives are not cytotoxic at the concentration required to inhibit AChE enzyme effectively.

3. Conclusion

The 2-hydroxybenzaldehyde-nicotinonitriles were synthesized in excellent yields. These hybrids were used as key intermediates for the preparation of coumarin derivatives. The new hybrids were evaluated for their AChE inhibitory efficacies. Coumarin hybrids with 6-(4-

chlorophenyl) and 6-(4-nitrophenyl) groups, linked to 4-phenylnicotinonitrile moiety, displayed more powerful efficacies than donepezil. Some coumarins exhibited strong free capacity to quench radicals against DPPH. Some new hybrids showed cytotoxicity in micromolar range.

4. Experimental

“All solvents were acquired from commercial sources and used as received unless otherwise stated. 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 ^1H 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”.^[36] 2-Thioxopyridine-3-carbonitriles **6(7)** were prepared according to literature procedures.^[42-44]

Preparation of nicotinonitriles **9(10)**.

A mixture of 2-thioxopyridine-3-carbonitriles **6(7)** (5 mmol) in ethanol (20 mL) containing KOH (5 mmol) was stirred at rt for 10 min. To the mixture, a solution of benzyl chloride derivative **8** (5 mmol) in 10 mL of ethanol was added and the stirring was continued for 2 h. The obtained nicotinonitriles were collected by filtration, washed with each of water, dried and recrystallized from dioxane / ethanol mixture.

2-((3-Formyl-4-hydroxybenzyl)thio)-4,6-diphenylnicotinonitrile (**9a**).

Colorless solid (91%); m.p. 114 °C; IR: ν 3422 (OH), 2236 (CN), 1679 (CO) cm^{-1} ; ^1H -NMR (DMSO- d_6): δ 4.63 (s, 2H, CH_2), 6.96 (d, 1H, ArH), 7.46 (t, 1H, ArH), 7.50 (t, 2H, ArH's), 7.54 (t, 1H, ArH), 7.59 (t, 2H, ArH's), 7.65 (d, 1H, ArH), 7.73 (d, 2H, ArH's), 7.80 (s, 1H, ArH), 7.83 (s, 1H, H5), 7.95 (d, 2H, ArH's), 10.22 (s, 1H, CHO), 10.67 (s, 1H, OH); ^{13}C -NMR (DMSO- d_6): δ 33.7, 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; MS (m/z):

422 (M^+ , 48.2%); Anal. for $C_{26}H_{18}N_2O_2S$: C, 73.91; H, 4.29; N, 6.63; found: C, 73.75; H, 4.41; N, 6.52%.

6-(4-Chlorophenyl)-2-((3-formyl-4-hydroxybenzyl)thio)-4-phenylnicotinonitrile (9b).

Colorless solid (90%); m.p. 122 °C; IR: ν 3425 (OH), 2230 (CN), 1677 (CO) cm^{-1} ; 1H -NMR (DMSO- d_6): δ 4.64 (s, 2H, CH_2), 6.96 (d, 1H, ArH), 7.50 (d, 2H, ArH's), 7.56 (t, 1H, ArH), 7.60 (t, 2H, ArH's), 7.66 (d, 1H, ArH), 7.78 (s, 1H, ArH), 7.81 (s, 1H, H5), 7.95 (d, 2H, ArH's), 8.12 (d, 2H, ArH's), 10.22 (s, 1H, CHO), 10.65 (s, 1H, OH); ^{13}C -NMR (DMSO- d_6): δ 33.4, 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; 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 (87%); m.p. 120 °C; IR: ν 3432 (OH), 2231 (CN), 1675 (CO) cm^{-1} ; 1H -NMR (DMSO- d_6): δ 2.38 (s, 3H, CH_3), 4.64 (s, 2H, CH_2), 6.96 (d, 1H, ArH), 7.33 (d, 2H, ArH's), 7.54 (t, 1H, ArH), 7.59 (t, 2H, ArH's), 7.64 (d, 1H, ArH), 7.79 (s, 1H, ArH), 7.82 (s, 1H, H5), 7.96 (d, 2H, ArH's), 8.14 (d, 2H, ArH's), 10.21 (s, 1H, CHO), 10.65 (s, 1H, OH); ^{13}C -NMR (DMSO- d_6): δ 21.5, 33.6 (CH_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; MS (m/z): 436 (M^+ , 38.0%); Anal. for $C_{27}H_{20}N_2O_2S$: C, 74.55; H, 4.69; N, 6.27; found: C, 74.27; H, 4.65; N, 6.40%.

2-((3-Formyl-4-hydroxybenzyl)thio)-6-(4-methoxyphenyl)-4-phenylnicotinonitrile (9d).

Colorless solid (93%); m.p. 184 °C; IR: ν 3414 (OH), 2231 (CN), 1674 (CO) cm^{-1} ; 1H -NMR (DMSO- d_6): δ 3.83 (s, 3H, CH_3), 4.64 (s, 2H, CH_2), 6.96 (d, 1H, ArH), 7.06 (d, 2H, ArH's), 7.52 (t, 1H, ArH), 7.57 (t, 2H, ArH's), 7.62 (d, 1H, ArH), 7.71 (s, 1H, ArH), 7.78 (s, 1H, H5), 7.95 (d, 2H, ArH's), 8.07 (d, 2H, ArH's), 10.22 (s, 1H, CHO), 10.63 (s, 1H, OH); ^{13}C -NMR (DMSO- d_6): δ 33.5, 55.3, 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; 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, 71.54; H, 4.28; N, 6.33%.

2-((3-Formyl-4-hydroxybenzyl)thio)-6-(4-nitrophenyl)-4-phenylnicotinonitrile (9e).

Colorless solid (94%); m.p. 132 °C; IR: ν 3425 (OH), 2230 (CN), 1677 (CO) cm^{-1} ; $^1\text{H-NMR}$ (DMSO- d_6): δ 4.66 (s, 2H, CH_2), 6.94 (d, 1H, ArH), 7.53 (t, 1H, ArH), 7.59 (t, 2H, ArH's), 7.64 (d, 1H, ArH), 7.80 (s, 1H, ArH), 7.84 (s, 1H, H5), 7.96 (d, 2H, ArH's), 8.05 (d, 2H, ArH's), 8.38 (d, 2H, ArH's), 10.22 (s, 1H, CHO), 10.65 (s, 1H, OH); $^{13}\text{C-NMR}$ (DMSO- d_6): δ 33.9, 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; MS (m/z): 467 (M^+ , 62.4%); Anal. for $\text{C}_{26}\text{H}_{17}\text{N}_3\text{O}_4\text{S}$: C, 66.80; H, 3.67; N, 8.99; found: C, 66.85; H, 3.83; N, 8.75%.

2-((3-Formyl-4-hydroxybenzyl)thio)-4-(4-methoxyphenyl)-6-phenylnicotinonitrile (10a).

Colorless solid (87%); m.p. 196 °C; IR: ν 3437 (OH), 2233 (CN), 1675 (CO) cm^{-1} ; $^1\text{H-NMR}$ (DMSO- d_6): δ 3.85 (s, 3H, CH_3), 4.66 (s, 2H, CH_2), 6.95 (d, 1H, ArH), 7.13 (d, 2H, ArH's), 7.53 (t, 1H, ArH), 7.57 (t, 2H, ArH's), 7.63 (d, 1H, ArH), 7.73 (d, 2H, ArH's), 7.78 (s, 1H, ArH), 7.81 (s, 1H, H5), 8.27 (d, 2H, ArH's), 10.20 (s, 1H, CHO), 10.68 (s, 1H, OH); $^{13}\text{C-NMR}$ (DMSO- d_6): δ 33.4, 55.9, 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; MS (m/z): 452 (M^+ , 74.6%); Anal. for $\text{C}_{27}\text{H}_{20}\text{N}_2\text{O}_3\text{S}$: C, 71.66; H, 4.45; N, 6.19; found: C, 71.78; H, 4.30; N, 6.07%.

6-(4-Chlorophenyl)-2-((3-formyl-4-hydroxybenzyl)thio)-4-(4-methoxyphenyl)nicotinonitrile (10b).

Colorless solid (93%); m.p. 132 °C; IR: ν 3422 (OH), 2233 (CN), 1676 (CO) cm^{-1} ; $^1\text{H-NMR}$ (DMSO- d_6): δ 3.83 (s, 3H, CH_3), 4.65 (s, 2H, CH_2), 6.95 (d, 1H, ArH), 7.10 (d, 2H, ArH's), 7.52 (d, 2H, ArH's), 7.65 (d, 1H, ArH), 7.71 (d, 2H, ArH's), 7.79 (s, 1H, ArH), 7.82 (s, 1H, H5), 8.16 (d, 2H, ArH's), 10.21 (s, 1H, CHO), 10.68 (s, 1H, OH); $^{13}\text{C-NMR}$ (DMSO- d_6): δ 33.5, 55.9, 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; Anal. for $\text{C}_{27}\text{H}_{19}\text{ClN}_2\text{O}_3\text{S}$ (486.9): C, 66.59; H, 3.93; N, 5.75; found: C, 66.47; H, 3.84; N, 5.83%.

2-((3-Formyl-4-hydroxybenzyl)thio)-4-(4-methoxyphenyl)-6-(*p*-tolyl)nicotinonitrile (10c).

Colorless solid (91%); m.p. 102 °C; IR: ν 3439 (OH), 2232 (CN), 1677 (CO) cm^{-1} ; $^1\text{H-NMR}$ (DMSO- d_6): δ 2.38 (s, 3H, CH_3), 3.84 (s, 3H, OCH_3), 4.65 (s, 2H, CH_2), 6.95 (d, 1H, ArH), 7.13 (d, 2H, ArH's), 7.34 (d, 2H, ArH's), 7.62 (d, 1H, ArH), 7.71 (d, 2H, ArH's), 7.80 (s, 1H, ArH), 7.82 (s, 1H, H5), 8.16 (d, 2H, ArH's), 10.21 (s, 1H, CHO), 10.68 (s, 1H, OH); $^{13}\text{C-NMR}$

NMR (DMSO-*d*₆): δ 21.4, 33.4, 55.9, 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; MS (*m/z*): 466 (M^+ , 44.5%); Anal. for C₂₈H₂₂N₂O₃S: C, 72.08; H, 4.75; N, 6.00; found: C, 72.26; H, 4.60; N, 6.07%.

2-((3-Formyl-4-hydroxybenzyl)thio)-4,6-bis(4-methoxyphenyl)nicotinonitrile (10d).

Colorless solid (91%); m.p. 122 °C; IR: ν 3431 (OH), 2228 (CN), 1671 (CO) cm⁻¹; ¹H-NMR (DMSO-*d*₆): δ 3.83 (s, 6H, 2 CH₃), 4.63 (s, 2H, CH₂), 6.95 (d, 1H, ArH), 7.05 (d, 2H, ArH's), 7.12 (d, 2H, ArH's), 7.67 (d, 1H, ArH), 7.75-7.79 (m, 4H, 3 ArH's and H5), 8.22 (d, 2H, ArH's), 10.21 (s, 1H, CHO), 10.69 (s, 1H, OH); ¹³C-NMR (DMSO-*d*₆): δ 32.8, 55.3 (2 C), 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; MS (*m/z*): 482 (M^+ , 70.4%); Anal. for C₂₈H₂₂N₂O₄S: C, 69.69; H, 4.60; N, 5.81; found: C, 69.74; H, 4.58; N, 5.71%.

2-((3-Formyl-4-hydroxybenzyl)thio)-4-(4-methoxyphenyl)-6-(4-nitrophenyl)nicotinonitrile (10e).

Colorless solid (93%); m.p. 130 °C; IR: ν 3427 (OH), 2232 (CN), 1679 (CO) cm⁻¹; ¹H-NMR (DMSO-*d*₆): δ 3.84 (s, 3H, CH₃), 4.64 (s, 2H, CH₂), 6.95 (d, 1H, ArH), 7.08 (d, 2H, ArH's), 7.62 (d, 1H, ArH), 7.78 (s, 1H, ArH), 7.80 (s, 1H, H5), 8.05 (d, 2H, ArH's), 8.25 (d, 2H, ArH's), 8.38 (d, 2H, ArH's), 10.22 (s, 1H, CHO), 10.65 (s, 1H, OH); ¹³C-NMR (DMSO-*d*₆): δ 33.8, 55.6, 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; MS (*m/z*): 497 (M^+ , 70.3%); Anal. for C₂₇H₁₉N₃O₅S: C, 65.18; H, 3.85; N, 8.45; found: C, 65.16; H, 3.88; N, 8.46%.

Preparation of N-(6-(((3-cyanopyridin-2-yl)thio)methyl)-2-oxo-2H-chromen-3-yl)benzamides 12(13).

A mixture of benzoylglycine **11** and 2-hydroxybenzaldehydes **9(10)** (5 mmol) in 20 mL of acetic anhydride containing 8 mmol of fused sodium acetate was heated at 100 °C. After heating the reaction for 6 h, the volume of the mixture was reduced to its half by evaporation. After cooling the mixture, the mixture product was filtered off, washed with ethanol and recrystallized from dioxane.

N-(6-(((3-Cyano-4,6-diphenylpyridin-2-yl)thio)methyl)-2-oxo-2H-chromen-3-yl)benzamide (12a).

Yellow solid (81%, cLogP 7.47); m.p. 224 °C; IR: ν 3338 (NH), 2230 (CN), 1692, 1654 (2 CO) cm^{-1} ; $^1\text{H-NMR}$ (DMSO- d_6): δ 4.70 (s, 2H, CH_2), 7.41 (d, 1H, H8), 7.46 (t, 1H, ArH), 7.50-7.54 (m, 3H, ArH's), 7.57-7.61 (m, 3H, ArH's), 7.64 (t, 2H, ArH's), 7.80 (s, 1H, pyridine-H), 7.84 (d, 1H, H7), 7.92-7.95 (m, 4H, ArH's), 8.06 (s, 1H, H5), 8.25 (d, 2H, ArH's), 8.32 (s, 1H, H4), 9.72 (br s, 1H, NH); $^{13}\text{C-NMR}$ (DMSO- d_6): δ 33.6, 103.8, 115.8, 116.4, 116.6, 118.3, 124.2, 126.6, 127.7, 127.9, 128.1, 128.3, 128.6, 129.2, 129.6, 129.8, 130.5, 131.5, 133.6, 135.3, 136.3, 136.5, 144.8, 152.7, 157.4, 157.9, 159.8, 161.2, 165.6; MS m/z (%): 565 (M^+ , 51.4); Anal. for $\text{C}_{35}\text{H}_{23}\text{N}_3\text{O}_3\text{S}$: C, 74.32; H, 4.10; N, 7.43; found: C, 74.17; H, 3.97; N, 7.58%.

***N*-(6-(((6-(4-Chlorophenyl)-3-cyano-4-phenylpyridin-2-yl)thio)methyl)-2-oxo-2*H*-chromen-3-yl)benzamide (12b).**

Yellow solid (84%, cLogP 8.03); m.p. 236-238 °C; IR: ν 3334 (NH), 2231 (CN), 1688, 1652 (2 CO) cm^{-1} ; $^1\text{H-NMR}$ (DMSO- d_6): δ 4.66 (s, 2H, CH_2), 7.40 (d, 1H, H8), 7.47 (d, 2H, ArH's), 7.50-7.54 (m, 2H, ArH), 7.57-7.61 (m, 4H, ArH's), 7.77 (s, 1H, pyridine-H), 7.81 (d, 1H, H7), 7.91 (d, 2H, ArH's), 7.95 (d, 2H, ArH's), 8.07 (s, 1H, H5), 8.12 (d, 2H, ArH's), 8.30 (s, 1H, H4), 9.64 (br s, 1H, NH); $^{13}\text{C-NMR}$ (DMSO- d_6): δ 33.7, 104.1, 115.7, 116.2, 116.4, 118.0, 124.7, 126.4, 127.9, 128.0, 128.2, 128.4, 129.1, 129.2, 129.7, 130.5, 131.6, 133.8, 135.1, 135.3, 135.8, 136.5, 146.3, 153.5, 158.0, 158.2, 160.0, 161.7, 165.8; Anal. for $\text{C}_{35}\text{H}_{22}\text{ClN}_3\text{O}_3\text{S}$ (600.0): C, 70.05; H, 3.70; N, 7.00; found: C, 70.24; H, 3.86; N, 6.84%.

***N*-(6-(((3-Cyano-4-phenyl-6-(*p*-tolyl)pyridin-2-yl)thio)methyl)-2-oxo-2*H*-chromen-3-yl)benzamide (12c).**

Yellow solid (79%, cLogP 7.96); m.p. 216-219 °C; IR: ν 3347 (NH), 2233 (CN), 1689, 1648 (2 CO) cm^{-1} ; $^1\text{H-NMR}$ (DMSO- d_6): δ 2.37 (s, 3H, CH_3), 4.71 (s, 2H, CH_2), 7.34 (d, 2H, ArH's), 7.39 (d, 1H, H8), 7.51-7.55 (m, 2H, ArH), 7.59-7.64 (m, 4H, ArH's), 7.81 (s, 1H, pyridine-H), 7.84 (d, 1H, H7), 7.90 (d, 2H, ArH's), 7.94 (d, 2H, ArH's), 8.02 (s, 1H, H5), 8.13 (d, 2H, ArH's), 8.27 (s, 1H, H4), 9.65 (br s, 1H, NH); $^{13}\text{C-NMR}$ (DMSO- d_6): δ 21.5, 33.6, 104.3, 115.9, 116.5, 116.7, 118.1, 124.7, 126.8, 127.8, 128.1, 128.4, 128.8, 129.0, 129.3, 130.9, 131.7, 133.5, 134.5, 135.3, 135.4, 135.8, 141.2, 146.6, 153.7, 158.2, 158.6, 160.1, 161.4, 165.6; MS (m/z): 579 (M^+ , 45.6%); Anal. for $\text{C}_{36}\text{H}_{25}\text{N}_3\text{O}_3\text{S}$: C, 74.59; H, 4.35; N, 7.25; found: C, 74.30; H, 4.52; N, 7.19%.

***N*-(6-(((3-Cyano-6-(4-methoxyphenyl)-4-phenylpyridin-2-yl)thio)methyl)-2-oxo-2*H*-chromen-3-yl)benzamide (12d).**

Yellow solid (74%, cLogP 7.34); m.p. 214 °C; IR: ν 3342 (NH), 2230 (CN), 1691, 1649 (2 CO) cm^{-1} ; $^1\text{H-NMR}$ (DMSO- d_6): δ 3.82 (s, 3H, CH₃), 4.67 (s, 2H, CH₂), 7.06 (d, 2H, ArH's), 7.41 (d, 1H, H8), 7.47-7.52 (m, 2H, ArH), 7.55-7.59 (m, 4H, ArH's), 7.82 (s, 1H, pyridine-H), 7.88 (d, 1H, H7), 7.91 (d, 2H, ArH's), 7.98 (d, 2H, ArH's), 8.10 (s, 1H, H5), 8.22 (d, 2H, ArH's), 8.38 (s, 1H, H4), 9.76 (br s, 1H, NH); $^{13}\text{C-NMR}$ (DMSO- d_6): δ 33.5, 55.3, 102.8, 114.4, 116.0, 116.4, 116.7, 119.2, 124.6, 125.8, 127.9, 128.1, 128.4, 129.0, 129.2, 129.5, 130.4, 130.7, 131.7, 133.8, 135.5, 136.4, 146.4, 155.2, 157.2, 158.3, 158.9, 160.2, 162.3, 165.5; MS m/z (%): 595 (M^+ , 42.4); Anal. for C₃₆H₂₅N₃O₄S: C, 72.59; H, 4.23; N, 7.05; found: C, 72.67; H, 4.18; N, 6.94%.

***N*-(6-(((3-Cyano-6-(4-nitrophenyl)-4-phenylpyridin-2-yl)thio)methyl)-2-oxo-2*H*-chromen-3-yl)benzamide (12e).**

Yellow solid (80%, cLogP 6.07); m.p. 244-247 °C; IR: ν 3349 (NH), 2233 (CN), 1694, 1653 (2 CO) cm^{-1} ; $^1\text{H-NMR}$ (DMSO- d_6): δ 4.71 (s, 2H, CH₂), 7.39 (d, 1H, H8), 7.45 (t, 1H, ArH), 7.49 (t, 1H, ArH), 7.53-7.58 (m, 4H, ArH's), 7.79 (s, 1H, pyridine-H), 7.82 (d, 1H, H7), 7.88 (d, 2H, ArH's), 7.93 (d, 2H, ArH's), 8.04 (s, 1H, H5), 8.23 (d, 2H, ArH's), 8.35 (s, 1H, H4), 8.42 (d, 2H, ArH's), 9.82 (br s, 1H, NH); $^{13}\text{C-NMR}$ (DMSO- d_6): δ 33.7, 103.7, 116.1, 116.9, 117.2, 119.6, 122.1, 124.6, 124.9, 125.7, 127.8, 128.1, 128.3, 129.1, 129.7, 130.6, 131.7, 133.6, 135.7, 136.6, 138.5, 144.6, 146.8, 156.0, 158.0, 158.6, 160.4, 161.8, 165.7; MS m/z (%): 610 (M^+ , 37.9); Anal. for C₃₅H₂₂N₄O₅S: C, 68.84; H, 3.63; N, 9.18; found: C, 69.04; H, 3.51; N, 9.03%.

***N*-(6-(((3-Cyano-4-(4-methoxyphenyl)-6-phenylpyridin-2-yl)thio)methyl)-2-oxo-2*H*-chromen-3-yl)benzamide (13a).**

Yellow solid (82%, cLogP 7.34); m.p. 216-219 °C; IR: ν 3349 (NH), 2232 (CN), 1689, 1642 (2 CO) cm^{-1} ; $^1\text{H-NMR}$ (DMSO- d_6): δ 3.83 (s, 3H, CH₃), 4.65 (s, 2H, CH₂), 7.11 (d, 2H, ArH's), 7.39 (d, 1H, H8), 7.49-7.54 (m, 2H, ArH's), 7.57-7.62 (m, 4H, ArH's), 7.73 (d, 2H, ArH's), 7.85 (s, 1H, pyridine-H), 7.89 (d, 1H, H7), 7.94 (d, 2H, ArH's), 8.11 (s, 1H, H5), 8.25 (d, 2H, ArH's), 8.38 (s, 1H, H4), 9.81 (br s, 1H, NH); $^{13}\text{C-NMR}$ (DMSO- d_6): δ 33.6, 55.6, 103.3, 114.5, 115.4, 115.9, 116.4, 118.5, 124.8, 126.2, 127.7, 127.9, 128.2, 128.8, 129.2, 129.4, 130.4, 130.6, 131.7, 133.6, 135.4, 136.3, 146.2, 153.0, 157.9, 158.3, 158.7,

160.3, 162.1, 165.6; MS (m/z): 595 (M^+ , 62.5%); Anal. for $C_{36}H_{25}N_3O_4S$: C, 72.59; H, 4.23; N, 7.05; found: C, 72.42; H, 4.08; N, 6.91%.

***N*-(6-(((6-(4-Chlorophenyl)-3-cyano-4-(4-methoxyphenyl)pyridin-2-yl)thio)methyl)-2-oxo-2*H*-chromen-3-yl)benzamide (13b).**

Yellow solid (75%, cLogP 7.90); m.p. 233 °C; IR: ν 3352 (NH), 2233 (CN), 1695, 1647 (2 CO) cm^{-1} ; 1H -NMR (DMSO- d_6): δ 3.84 (s, 3H, CH_3), 4.72 (s, 2H, CH_2), 7.10 (d, 2H, ArH's), 7.41 (d, 1H, H8), 7.52 (t, 1H, ArH), 7.56-7.61 (m, 4H, ArH's), 7.78 (s, 1H, pyridine-H), 7.83 (d, 1H, H7), 7.96 (d, 2H, ArH's), 8.09 (s, 1H, H5), 8.14 (d, 2H, ArH's), 8.24 (d, 2H, ArH's), 8.29 (s, 1H, H4), 9.62 (br s, 1H, NH); ^{13}C -NMR (DMSO- d_6): δ 33.7, 55.6, 103.5, 114.6, 116.4, 116.5, 116.9, 118.4, 124.9, 126.3, 128.0, 128.1, 128.8, 129.1, 129.3, 130.6, 130.8, 131.4, 133.6, 135.2, 135.3, 135.9, 146.4, 153.8, 158.2, 158.6, 160.2, 160.4, 161.9, 165.5; Anal. for $C_{36}H_{24}ClN_3O_4S$ (630.1): C, 68.62; H, 3.84; N, 6.67; found: C, 68.76; H, 3.99; N, 6.54%.

***N*-(6-(((3-Cyano-4-(4-methoxyphenyl)-6-(*p*-tolyl)pyridin-2-yl)thio)methyl)-2-oxo-2*H*-chromen-3-yl)benzamide (13c).**

Yellow solid (85%, cLogP 7.83); m.p. 218-220 °C; IR: ν 3336 (NH), 2230 (CN), 1687, 1641 (2 CO) cm^{-1} ; 1H -NMR (DMSO- d_6): δ 2.36 (s, 3H, CH_3), 3.84 (s, 3H, OCH_3), 4.69 (s, 2H, CH_2), 7.11 (d, 2H, ArH's), 7.34 (d, 2H, ArH's), 7.38 (d, 1H, H8), 7.52 (t, 1H, ArH), 7.57 (t, 2H, ArH's), 7.71 (d, 2H, ArH's), 7.79 (s, 1H, pyridine-H), 7.82 (d, 1H, H7), 7.95 (d, 2H, ArH's), 8.02 (s, 1H, H5), 8.13 (d, 2H, ArH's), 8.33 (s, 1H, H4), 9.62 (br s, 1H, NH); ^{13}C -NMR (DMSO- d_6): δ 21.5, 33.8, 55.7, 103.2, 114.5, 115.9, 116.2, 116.4, 117.9, 125.2, 126.4, 127.7, 128.0, 128.6, 128.8, 129.2, 130.4, 130.6, 131.6, 133.8, 134.6, 135.3, 141.4, 145.9, 155.2, 157.6, 157.8, 159.9, 160.5, 161.8, 165.3; MS (m/z): 609 (M^+ , 61.3%); Anal. for $C_{37}H_{27}N_3O_4S$: C, 72.89; H, 4.46; N, 6.89; found: C, 73.07; H, 4.56; N, 7.00%.

***N*-(6-(((3-Cyano-4,6-bis(4-methoxyphenyl)pyridin-2-yl)thio)methyl)-2-oxo-2*H*-chromen-3-yl)benzamide (13d).**

Yellow solid (76%, cLogP 7.22); m.p. 204-206 °C; IR: ν 3347 (NH), 2231 (CN), 1691, 1645 (2 CO) cm^{-1} ; 1H -NMR (DMSO- d_6): δ 3.81 (s, 3H, CH_3), 3.85 (2 s, 3H, CH_3), 4.64 (s, 2H, CH_2), 7.05 (d, 2H, ArH's), 7.10 (d, 2H, ArH's), 7.43 (d, 1H, H8), 7.51 (t, 1H, ArH), 7.55 (t, 2H, ArH's), 7.66 (d, 2H, ArH's), 7.76 (s, 1H, pyridine-H), 7.81 (d, 1H, H7), 7.93 (d, 2H, ArH's), 8.04 (s, 1H, H5), 8.15 (d, 2H, ArH's), 8.29 (s, 1H, H4), 9.70 (br s, 1H, NH); ^{13}C -

NMR (DMSO-*d*₆): δ 33.8, 55.8 (2 C), 101.8, 114.2, 114.6, 115.3, 115.8, 116.5, 118.0, 124.7, 125.8, 127.8, 128.2, 129.0, 129.4, 130.2, 130.4, 130.8, 131.4, 133.5, 135.4, 145.7, 153.8, 157.6, 157.8, 158.4, 160.2, 160.3, 161.6, 165.5; MS (*m/z*): 625 (M^+ , 33.8%); Anal. for C₃₇H₂₇N₃O₅S: C, 71.03; H, 4.35; N, 6.72; found: C, 70.84; H, 4.51; N, 6.63%.

***N*-(6-(((3-Cyano-4-(4-methoxyphenyl)-6-(4-nitrophenyl)pyridin-2-yl)thio)methyl)-2-oxo-2*H*-chromen-3-yl)benzamide (13e).**

Yellow solid (76%, cLogP 6.20); m.p. 240-241 °C; IR: ν 3346 (NH), 2232 (CN), 1688, 1651 (2 CO) cm⁻¹; ¹H-NMR (DMSO-*d*₆): δ 3.83 (s, 3H, CH₃), 4.71 (s, 2H, CH₂), 7.09 (d, 2H, ArH's), 7.42 (d, 1H, H₈), 7.55 (t, 1H, ArH), 7.59 (t, 2H, ArH's), 7.76 (s, 1H, pyridine-H), 7.81 (d, 1H, H₇), 7.93 (d, 2H, ArH's), 8.08 (s, 1H, H₅), 8.22 (d, 2H, ArH's), 8.27 (d, 2H, ArH's), 8.33 (s, 1H, H₄), 8.39 (d, 2H, ArH's), 9.82 (br s, 1H, NH); ¹³C-NMR (DMSO-*d*₆): δ 33.8, 55.8, 102.6, 114.6, 115.5, 115.9, 116.3, 117.8, 122.3, 124.6, 124.7, 126.2, 127.8, 128.0, 129.3, 130.2, 130.7, 131.8, 133.9, 135.4, 138.7, 144.6, 146.3, 154.2, 157.9, 158.1, 160.2, 160.5, 161.9, 165.6; MS (*m/z*): 640 (M^+ , 32.7%); Anal. for C₃₆H₂₄N₄O₆S: C, 67.49; H, 3.78; N, 8.75; found: C, 67.32; H, 3.92; N, 8.83%.

4.3. AChE inhibition assay

“AChE inhibitory activities were assessed using Ellman procedure^[48] with minor changes. Stock solutions of the tested hybrids were prepared in a mixture of 1 mL DMSO and 9 mL methanol, followed by dilution in the buffer KH₂PO₄/K₂HPO₄ (50 mM, pH 7.7) to acquire the final concentration. To 60 μ L of 50 mM phosphate buffer (pH 7.7), 10 μ L of the respective assayed sample (at stock solution of 0.5 mM) was applied. Then, 10 μ L 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 applied. The resulting content was mixed and pre-read at 405 nm, then incubated at 37 °C for 10 min. In each well, the reaction was started by adding 10 μ L of acetylthiocholine iodide (0.5 mM), followed by adding 10 μ L of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB, 0.5 mM per well). At 37 °C, the wells were incubated, 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:”^[55]

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

Each compound was tested in triplicates at concentration of 25 nM.

4.4. DPPH radical-scavenging assay

“DPPH radical-scavenging activity was assessed according to Thuong et. al. procedure with minor changes. Methanol was used as a solvent to assess the tested derivatives. 1 mL of methanolic DPPH solution (0.3 mM) was applied to 2.5 mL of the tested derivative in 96-well plates. 1 mL of methanol was applied and the solution was mixed for a minute at rt and incubated in a dark place. After 30 min, the absorbance of the reaction mixture was calculated at 520 nm on a microplate reader. The reading blank consisted of 2.5 mL of estimated hybrid 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:”^[56]

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

The new hybrids as well as the reference ascorbic acid have been examined in triplicates at concentration of 25 µg/mL.

4.5. Cytotoxicity

4.5.1. 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.”^[57]

“The selected cell lines were cultivated in Dulbecco’s Modified Eagle’s Medium (DMEM). All of the growth media were supplemented with 10% Foetal Bovine Serum (FBS) and antibiotics (100 U/mL penicillin and 100 mg/mL streptomycin) at 37°C in a humidified atmosphere containing 5% CO₂. 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.”^[58]

4.5.2. Neutral red uptake assay (NRU assay)

“The NRU assay depends on the ability of living cells to bind neutral red, in lysosomes.^[59] 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 counted using hemocytometer, and cell viability 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 μ L of the cell suspension was dispensed by multichannel pipette into the inner 60 wells of the 96 well plate, the peripheral wells were filled with PBS, then the plate 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 μ g/mL) were prepared using DMEM media. 200 μ L 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 incubated at 37° C for 48 h. Then, the medium and extracts were discarded and replaced with 100 μ L 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 μ L 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 were 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.”^[58]

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