Synthesis and anticancer evaluation of some coumarin and azacoumarin derivatives

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Coumarin and its nitrogen analogue 1-aza coumarin are a class of lactones and lactams, respectively, which are indispensable heterocyclic units to both chemists and biochemists. 1-Aza coumarin derivatives, which ultimately metabolize as the corresponding 8-hydroxy coumarins in the biological system are therefore found to be very good anti-inflammatory, anti-cancer, and analgesic agents. A series of hybrid substituted coumarin and azacoumarin-3-carboxylic acid derivatives (8-methoxycoumarin-3-carboxylic acid (4a), 8-methoxazacoumarin-3-carboxylic acid (4b), 5-bromo-8-methoxycoumarin-3-carboxylic acid (5a), 5-bromo-8-methoxazacoumarin-3-carboxylic acid (5b), 2-acetoxy-5-bromo-8-methoxyquinoline-3-carboxylic acid (6), and 5,7-di(phenylazo)-8-methoxycoumarin-3-carboxylic acid (7) were synthesized and structurally proved using spectral and elemental analysis data. Substituted coumarin-3-carboxylic acid (4a and 5a) and Substituted azacoumarin-3-carboxylic acid (4b, 5b and 6) were tested for their in vitro cytotoxic activity against MCF-7 and HepG-2 cell lines.

Keywords: Coumarin; azacoumarin; 1H-NMR spectrum; 13C-NMR spectrum; anticancer.

INTRODUCTION

The chemistry of coumarin and its derivatives play an important role in the agricultural and pharmaceutical industries. The parent coumarin was first isolated in 1820 by Vogel from tanka beans1. Recently, about 150 species of coumarin derivatives have been found in 30 diverse plant families, such as Rutaceae, Apiaceae, Asteraceae, Leguminosae, Glusiaeae, Guttiferae, Oleacea, Umbelliferae2, as well as occurs as animal and microbial metabolites3. Its diverse pharmacological effects including antimicrobial4,5, antioxidant6, anti-HIV7,8, anticoagulant9, antihyperensive10, antitubercular11, anticonvulsant12, antihyperglycemic and anticancer13-16. Cancer is one of the main health problems in the overall world. As a result of the increased number of deaths due to cancer and estimated with 9.6 million death in 201817,18. Multiple proteins and enzymes are deregulated in this devasting disease-causing difficulties for treatment with one target-based chemotherapeutics. The initiation and progression of this complex disease depend on numerous receptors or signaling pathways indicating that multi-targeted therapy could have prominent efficacy as compared to solo-targeting therapy19-24. This can be achieved by using the hybridization technique. In continuous efforts to fight against this tremendous problem, various research groups have developed coumarin-based hybrid molecules25-29.

The activity of coumarins as anticancer agents is at the top of reviews published in 202030-33, as well as research papers. Potent inhibitors of aldo–keto reductase (AKR) presenting an iminocoumarin scaffold, with activities between 25 and 56 nM, have been described for the treatment of prostatic cancer34. The design of sulfamide 3-benzylcoumarin hybrids bearing an oxadiazole ring at position 7 has allowed the preparation of new multitarget mitogen-activated protein kinase (MEK) inhibitors and nitric oxide (NO) donors, both with antiproliferative properties35. In other cases, the anticancer profile has been directed to other targets. Such is the case of new inhibitors of cyclin-dependent kinases, specifically CDK9, designing hybrids that incorporate an aminopyrimidine fragment to coumarin, both pharmacophores of known activity on this therapeutic targets36. Within the group of compounds with anticancer activity, coumarins exhibiting an antigloma profile may be highlighted. Simple coumarins such as osthole, umbellifer-one, esculin, and 4-hydroxycoumarin, combined with sorafenib (a kinase inhibitor drug approved for the treatment of primary kidney cancer, advanced primary liver cancer, FLT3-ITD positive acute myeloid leukemia (AML), and radioactive iodine-resistant advanced thyroid carcinoma) were studied37. The same group also studied a combination of the same simple coumarins with temozolomide (used in the treatment of brain tumors such as glioblastoma multiforme or anaplastic astrocytoma)38.

This paper reported the synthesis of some hybrid coumarin-3-carboxylic acid and azacoumarin-3-carboxylic acid derivatives using aromatic aldehyde derivatives (1) as key starting material. Some of the synthesized hybrid coumarin-3-carboxylic acid and azacoumarin-3-carboxylic acid derivatives are evaluated in vitro against human tumor cell lines.

EXPERIMENTAL

Instrumental analysis

The IR data were obtained on a Shimadzu 470 spectrometer. The melting point of the synthesized compounds was determined with an electrothermal melting point apparatus and has not been corrected. 1H-NMR (400 MHz) and 13C-NMR (100 MHz) spectra were run with a Bruker 400 DRX-Avance NMR spectrometer. The compounds were dissolved in deuterated DMSO as a solvent. The molecular weight of the compounds was determined by an electron ionization (EI) mass spectrometer performed using a Probe Agilent MSD-5975 spectrometer operating at 70 eV. The elemental analysis was performed on a Perkin-Elmer 2400 series II CHN elemental analyzer. All reagents were obtained from Aldrich chemical company and used as supplied.
Chemistry

General procedure for the synthesis of ethyl coumarin and/or azacoumarin-3-carboxylate 2

Compounds 2a and 2b were obtained by fusion of the mixture of aromatic aldehydes (namely, 3-methoxy-2-hydroxybenzaldehyde and 3-methoxy-2-aminobenzaldehyde (0.01 mol) and diethyl malonate (0.01 mol) in the presence of piperidine (2 mL) on a hot plate for 2–3 min, ethanol (50 mL) was added to the reaction mixture and heated under reflux for 2 h, then cooled and poured into ice-water. The reaction mixture was neutralized with dilute hydrochloric acid (2%) and the resulting solid was filtered off, washed with water and dried. Finally, the product was crystallized from benzene to give 2.

Ethyl 8-methoxycoumarin-3-carboxylate (2a)

As colorless crystals yield 82%. m.p. 105–107°C. IR (KBr) ν max: 1735, 1715 (C=O), 1605, 1585 (C=C), 1125, 1083 (C-O) cm –1. 1H-NMR (DMSO-d6): 0.87 (t, 3H, CH3), 3.87 (s, 3H, OCH3), 4.23 (q, 2H, OCH2), 7.21–7.52 (m, 3H, Ar-H), 8.56 (s, 1H, H-4 of pyranone ring) ppm. Anal. Calcd. For C13H12O5 (248): C, 62.90; H, 4.84. Found: C, 62.62; H, 4.49.

Ethyl 8-methoxyazacoumarin-3-carboxylate (2b)

As pale-yellow crystals yield 76%. m.p. 123–125°C. IR (KBr) ν max: 3289 (NH), 1745, 1689 (C=O), 1610, 1578 (C=C), 1095, 1062 (C-O) cm –1. 1H-NMR (DMSO-d6): 1.37 (t, 3H, CH3), 3.87 (s, 3H, OCH3), 4.23 (q, 2H, OCH2), 7.31–7.52 (m, 3H, Ar-H), 8.11 (br. s, 1H, NH) ppm. MS: m/z (%) = 300 (M++2), 299 (M++1, 21.82), 298 (M+, 100), 283 (15.08), 281 (6.73), 279 (16.04), 278 (68.04), 276 (68.30), 254 (67.56), 242 (64.99), 241 (23.03), 240 (4.02), 239 (24.68), 229 (5.53), 228 (20.78), 227 (10.25), 226 (22.62), 220 (4.02), 219 (5.09), 218 (1.24), 214 (6.73), 213 (68.04), 211 (68.30), 200 (4.48), 199 (9.19), 198 (3.83), 197 (8.73), 196 (6.81), 191 (9.91), 186 (4.49), 185 (46.51), 184 (7.38), 183 (49.85), 176 (9.81), 175 (28.37), 173 (8.64), 171 (4.59), 170 (3.04), 169 (12.44), 167 (11.54), 163 (5.46), 161 (2.50), 159 (3.94), 157 (30.22), 156 (7.29), 155 (30.90), 154 (5.54), 148 (6.74), 147 (23.08), 145 (41.14), 133 (5.36), 132 (10.07), 120 (17.69), 119 (17.52), 118 (6.77), 117 (7.84), 105 (3.83), 104 (8.74), 103 (40.40), 102 (4.82), 101 (5.97), 92 (10.57), 91 (8.08), 89 (14.51), 87 (15.93) 86 (11.08), 77 (13.69), 76 (33.01), 75 (66.08), 74 (44.03), 65 (4.28), 64 (5.80), 63 (17.74), 62 (13.88), 61 (8.07), 53 (12.88), 51 (6.26), 50 (9.14). Anal. Calcd. For C11H11NO3 (247): C, 63.16; H, 5.26; N, 5.67. Found: C, 62.96; H, 5.03; N, 5.35.

General procedure for the synthesis of 5-bromo-8-methoxycoumarin-3-carboxylic acid (5)

In 20 mL of glacial acetic acid, acid derivative 4 (0.01 mol) was dissolved, then 10 mL of bromine (0.01 mol) in glacial acetic acid was added drop wise to compound 4 with stirring at 40–50°C. After 5–10 min the bromine color was discharged and yellow solution remained. At this point, 0.5–1.0 mL of bromine-AcOH solution was added with stirring at room temperature for 2 h. The reaction mixture was poured unto water with stirring, and the solid formed was filtered off, washed with water, and dried. Finally, the product was crystallized from ethanol to give 5.

5-bromo-8-methoxycoumarin-3-carboxylic acid (5a)

As pale-yellow crystals yield 73%. m.p. 215–217°C. IR (KBr) ν max: 3380–2850 (br. OH), 1720–1705 (C=O), 1613, 1591 (C=C), 1171, 1093 (C-O) cm –1. 1H-NMR (DMSO-d6): 3.90 (s, 3H, OCH3), 7.33 (d, 1H, Ar-H), 7.61 (d, 1H, Ar-H), 8.55 (s, 1H, H-4 of coumarin ring) ppm. 13C-NMR (DMSO-d6): 163.51 (C=O), 155.39, 146.14, 146.07, 144.85, 128.03, 119.69, 115.73, 116.95, 112.03, 56.40 (OCH3) ppm. MS: m/z (%): 300 (M++2), 299 (M++1, 21.82), 298 (M+, 100), 283 (15.08), 281 (15.19), 257 (12.17), 256 (62.42), 255 (13.48), 254 (67.56), 242 (2.92), 241 (23.03), 240 (4.02), 239 (24.68), 229 (5.53), 228 (20.78), 227 (10.25), 226 (22.62), 220 (4.02), 219 (5.09), 218 (1.24), 214 (6.73), 213 (68.04), 211 (68.30), 200 (4.48), 199 (9.19), 198 (3.83), 197 (8.73), 196 (6.81), 191 (9.91), 186 (4.49), 185 (46.51), 184 (7.38), 183 (49.85), 176 (9.81), 175 (28.37), 173 (8.64), 171 (4.59), 170 (3.04), 169 (12.44), 167 (11.54), 163 (5.46), 161 (2.50), 159 (3.94), 157 (30.22), 156 (7.29), 155 (30.90), 154 (5.54), 148 (6.74), 147 (23.08), 145 (41.14), 133 (5.36), 132 (10.07), 120 (17.69), 119 (17.52), 118 (6.77), 117 (7.84), 105 (3.83), 104 (8.74), 103 (40.40), 102 (4.82), 101 (5.97), 92 (10.57), 91 (8.08), 89 (14.51), 87 (15.93) 86 (11.08), 77 (13.69), 76 (33.01), 75 (66.08), 74 (44.03), 65 (4.28), 64 (5.80), 63 (17.74), 62 (13.88), 61 (8.07), 53 (12.88), 51 (6.26), 50 (9.14). Anal. Calcd. For C11H7BrO4 (298): C, 44.29; H, 2.35. Found: C, 44.07; H, 2.22.
5-bromo-8-methoxycoumarin-3-carboxylic acid (5b)

As pale-yellow crystals yield 76%. m.p. 245–247°C. IR (KBr) νmax : 3398 (br. OH), 1615–1685 (br. C=O), 1618, 1581 (C=C), 1126, 1107, 1092 (C-O) cm⁻¹. 1H-NMR (DMSO-d₆) δ: 3.94 (s, 6H, 2OCH₃), 7.35–7.68 (m, 4H, Ar-H), 7.95 (br. s, 1H, NH), 8.09 (br. s, 2H, OH), 8.75 (s, 1H, H-4 of enol form of quinoline), 8.84 (s, 1H, H-4 of keto form of quinoline) ppm. 13C-NMR (DMSO-d₆) ppm: 119.83, 119.44, 117.32, 116.50, 112.64, (carbons of two isomers of quinoline ring), 56.92, 56.66 (2OCH₃) ppm.

Synthesis of 2-acetoxy-5-bromo-8-methoxyquinoline-3-carboxylic acid (6)

A solution of compound 5b (0.01 mol) in acetic anhydride (25 mL) was heated under reflux for 2 h, and then cooled. The reaction mixture was poured into ice-water with stirring and left for 24 h. The solid product was filtered off, washed with water, dried, and crystallized from ethanol to give 6.

As colorless crystals yield 61%. m.p. 155–157°C. IR (KBr) νmax : 3398 (br. OH), 1733–1715 (C=O), 1635 (C=N), 1608, 1582 (C=C), 1127, 1098, 1071 (C-O) cm⁻¹. 1H-NMR (DMSO-d₆) δ: 2.33 (s, 3H, COCH₃), 3.96 (s, 3H, OCH₃), 7.41 (d, 1H, Ar-H), 7.70 (d, 1H, Ar-H), 8.53 (s, 1H, H-4 of quinoline ring), 11.10 (s, 1H, OCH₃) ppm. 13C-NMR (DMSO-d₆) δ: 171.71, 162.31 (C=O), 158.77 (N=O=C), 146.77, 144.64, 144.60, 129.13, 123.00, 118.39, 117.68, 112.58, (carbons of quinoline ring), 57.00 (OCH₃) ppm. MS: m/z (%) = 339 (M⁺, unstable), 259 (1.87), 250 (2.22), 248 (2.63), 225 (13.59), 199 (1.58), 181 (56.82), 156 (2.67), 135 (2.63), 133 (9.70), 117 (27.09), 83 (4.99), 81 (14.58), 76 (1.12), 75 (3.02), 73 (1.02), 69 (64.27), 67 (1.87), 60 (15.85), 59 (4.67), 57 (40.77), 55 (100), 54 (10.44), 53 (22.99). Anal.Calcd. For C₁₃H₁₀NBrO₅: C, 46.02; H, 2.95; N, 4.53.

Synthesis of 5,7-di(phenylazo)-8-methoxycoumarin-3-carboxylic acid (7)

A solution of 8-methoxycoumarin-3-carboxylic acid (4a) (0.01 mol) in acetic anhydride (25 mL) was heated under reflux for 2 h, and then cooled. The reaction mixture was poured into ice-water with stirring and left for 24 h. The solid product was filtered off, washed with water, dried, and crystallized from ethanol to give 7.

As colorless crystals yield 61%. m.p. 155–157°C. IR (KBr) νmax : 3398 (br. OH), 1733–1715 (C=O), 1635 (C=N), 1608, 1582 (C=C), 1127, 1098, 1071 (C-O) cm⁻¹. 1H-NMR (DMSO-d₆) δ: 2.33 (s, 3H, COCH₃), 3.96 (s, 3H, OCH₃), 7.35–7.68 (m, 11H, Ar-H), 8.71 (s, 1H, H-4 of coumarin ring) ppm. 13C-NMR (DMSO-d₆) δ: 163.98 (C=O of carboxylic acid), 156.40 (C=O of coumarin), 152.10, 148.62 (C=O), 146.22, 143.81 (C-N), 129.42, 129.37, 129.30, 129.18, 124.73, 124.44, 122.34, 121.34, 121.71, 121.10, 118.49, 118.46, 116.21 (Carbons of aromatic and coumarin rings), 56.16 (OCH₃) ppm. MS: m/z (%) = 428 (M⁺, unstable), 247 (0.57), 273 (1.40), 258 (0.23), 257 (0.12), 255 (0.15), 254 (0.50), 221 (6.29), 220 (15.88), 204 (1.16), 203 (10.12), 198 (24.09), 197 (100), 196 (1.29), 177 (2.41), 176 (13.64), 175 (1.44), 169 (5.68), 168 (5.05), 167 (6.11), 166 (1.37), 149 (2.12), 148 (5.39), 147 (2.91), 146 (1.12), 141 (2.73), 134 (1.02), 133 (7.36),121 (4.08), 120 (42.94), 119 (1.94), 118 (1.89), 106 (1.08), 105 (9.97), 103 (1.32), 102 (1.30), 93 (11.84), 92 (45.17), 91 (3.66), 77 (23.20), 76 (2.20), 75 (1.44), 74 (1.47), 66 (3.77), 65 (19.79), 64 (2.65), 63 (3.77), 52 (2.43), 51 (7.61). Anal.Calcd. For C₃₂H₃₆N₂O₄: 428: C, 64.48; H, 3.74; N, 13.08. Found: C, 64.26; H, 3.55; N, 12.78.

Biological Evaluation

Cell Cultures

A human hepatocellular carcinoma (HepG2), human breast cancer (MCF-7) was propagated in RPMI-1640 medium L-Glutamine (Lonza Verviers SPRL, Belgium, cat#12-604F), and supplemented with 10% fetal bovine serum (FBS) (Seralab, UK, cat# EU-000-H), and 1% antibiotic (Antibiotic antimycotic, Biowest, cat#). The cells were incubated in 5% CO₂ humidified at 37°C for growth.

Evaluation of cell proliferation by MTT assay

The cytotoxic effect of the tested compounds on two cancer cell lines was evaluated by the MTT (3-[4,5-methythiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) assay as reported previously with slight modification. In brief, after evaluation of cell count and viability by trypan blue dye, cancer cells (1 x 10⁴ cells/well) were seeded in a 96-well plate in triplicate and could adhere for 24 h. The tested compounds were dissolved in 500 µl Dimethyl sulfoxide (DMSO) to have a stock solution of 100 mM, as the final concentration of DMSO in the culture medium never exceeded 0.2% (v/v) and then various concentrations of tested compounds were prepared by further diluting in complete medium to have a final concentration of (0.1, 1, 10, and 100 µM). In the next day, the medium was replaced with fresh medium with the indicated concentrations of tested compounds and cells were allowed to grow for 48 h. Four hours before completion of incubation, 10 µl of MTT (5 mg/ml in PBS w/o Ca, Mg, Lonza Verviers SPRL Belgium, cat#17-516F) was added in each well. After completing the incubation, 100 µl of Dimethyl sulfoxide (DMSO) was added to each well, then the 96 well plates were centrifuged for 5 minutes at 4000 rpm to precipitate the formazan crystals. The color developed after the reaction was measured at 490 nm using Bio-Tekmicroplate reader. The experiment was conducted in triplicate.

Data were calculated as a percent of cell viability by the following formula: % cell viability = (Mean absorbance in test wells / Mean absorbance in control wells) x 100. The effect of tested compounds on the morphology of treated lung cancer cells was investigated by the light microscope and then photographed by SONY CYBER-SHORT.
RESULTS AND DISCUSSION

Synthesis

The synthetic route to hybrid molecules containing coumarin-3-carboxylic acid and azacoumarin-3-carboxylic acid derivatives are presented in Scheme 1 and 2.

Ethyl 8-methoxycoumarin-3-carboxylate (2a) and ethyl 8-methoxyazacoumarin-3-carboxylate (2b) were obtained via the condensation of 3-methoxy-2-hydroxybenzaldehyde (1a) and 3-methoxy-2-amino benzaldehyde (1b) with diethyl malonate in the presence of piperidine as catalyst according to literature reported\textsuperscript{39, 40}.

The reaction of ester derivatives (2) with resorcinol in the presence of potassium hydroxide in methanol under reflux was expected to give structure 3, but only 8-methoxycoumarin-3-carboxylic acid (4a, known) and 8-methoxyazacoumarin-3-carboxylic acid (4b) are yielded (Scheme 1).

Structure of compound 4 was confirmed via its transformation into 8-methoxy-5-bromocoumarin-3-carboxylic acid (5a) and 8-methoxy-5-bromoazacoumarin-3-carboxylic acid (5b) via halogenation of compound 4 with bromine. Acetylation of 8-methoxy-5-bromoazacoumarin-3-carboxylic acid (5b) led to the formation of 8-methoxy-5-bromo-2-acetoxyquinoline-3-carboxylic acid (6)\textsuperscript{41}.

Diazotization of aniline followed by coupling with the sodium salt of 8-methoxycoumarin-3-carboxylic acid (4a) gave the corresponding 5,7-di(phenylazo)-8-methoxycoumarin-3-carboxylic acid (7, Scheme 2). The compound 4 contains free methoxy as electron-donating group at position 8, led to the substitution reaction with electrophilic reagents in the position 5 and 7 in this compound.

NMR spectra investigation of hybrid molecules (4–7)

To establish the structural feature of the synthesized compounds, \textsuperscript{1}H-NMR and \textsuperscript{13}C-NMR spectra of the hybrid 8-methoxycoumarin-3-carboxylic acid and 8-methoxyazacoumarin-3-carboxylic acid derivatives (4–7).

NMR spectra investigation of compounds 4a, 5a, and 7

The \textsuperscript{1}H-NMR spectrum of compound 4a showed sharp two singlet signals at $\Delta$ 3.90 and 8.71 ppm due to the protons of methoxy group and H-4 of coumarin ring, respectively. In addition, the \textsuperscript{1}H-NMR spectrum of compound 4a showed multiple signals in the region of $\Delta$ 7.32–7.43 ppm with the presence of three aromatic protons for the coumarin ring. The \textsuperscript{13}C-NMR spectrum of compound 4a showed two signals at $\Delta$ 163.998 and 156.40 ppm due to carbons of the carbonyl groups of carboxylic acid and coumarin ring. The \textsuperscript{13}C-NMR spectrum of compound 4a showed one signal at $\Delta$ 56.16 ppm assigned to the carbon of methoxy group. In addition, the \textsuperscript{13}C-NMR spectrum displayed 8 signals in the region of $\Delta$ 148.62–116.20 ppm due to the carbons of the coumarin ring.

Scheme 1. Synthesis of hybrid 8-methoxycoumarin-3-carboxylic (4a) and 8-methoxyazacoumarin-3-carboxylic acid (4b)
The 1H-NMR spectrum of compound 5a exhibited two singlet signals at 3.90 and 8.55 ppm for the protons of methoxy group (OCH₃) and H-4 of coumarin ring, and two double doublet signals at δ7.61 and 7.33 ppm assigned to H-6 and H-7 for the coumarin ring.

The 13C-NMR spectrum of compound 5a revealed characteristic carbon signals at δ163.51, 155.39 and 56.40 ppm refer to the carbon of carbonyl groups of carboxylic acid, coumarin and methoxy group (OCH₃), respectively. Also, the spectrum of these compounds showed eight carbon signals in the region at δ146.14–112.03 ppm due to the carbons of the coumarin ring.

From the study, the 1H-NMR spectrum of compound 7 showed that the presented two characteristic two singlet signals at δ3.90 and 8.71 ppm refer to the protons of methoxy group (OCH₃) and H-4 of coumarin ring protons of the aromatic ring in the compound 7 were observed within the expected chemical shift in the region of δ6.64–7.93 ppm as multiplet signals and exhibited the expected integral values.

The 13C-NMR spectrum of compound 7 showed the presented of three carbon signals at δ163.51, 155.39 and 56.40 ppm refer to the two carbonyl groups of acid, coumarin ring and methoxy group. Moreover, the 13C-NMR spectrum of compound 7 revealed characteristic new carbon signals in the region δ152.10–116.21 ppm equal to 16 signals due to the carbons of aromatic rings and C-3, C-4 of pyranone.

NMR spectra investigation of compounds 4b, 5b, and 6

The data of 1H-NMR spectrum of compound 4b gave clear cut evidence of two singlet signals at 3.91 and 8.82 ppm of protons for methoxy group (OCH₃) and H-4 of azacoumarin ring. Also, compound 4b containing the protons of carboxylic acid and NH group, which appeared in the 1H-NMR spectrum as broad singlet signals at δ8.10 and 7.96 ppm. three protons of the aromatic in the compound 4b were observed in the region of δ7.34–7.49 ppm as multiplet signals.

The 13C-NMR spectrum of compound 4 showed the presence of three carbon signals at δ162.48, 160.03 and 56.17 ppm refer to the two carbonyl groups of acid, amid, and carbon of methoxy (OCH₃). Also, the spectrum of this compound showed eight carbon signals in the region of δ148.01–116.00 ppm due to the carbons of the quinolinone ring.

From the study, the 1H-NMR spectrum of compound 5b showed the structure of these compound in keto-enol tautomers as shown in Figure 1.

The 1H-NMR spectrum of compound 5b showed that the presented two characteristic singlet signals at δ8.84 and 8.75 ppm refer to the keto form (40%) and enol form (60%). This occurred when both structures are unequilibrium states (40:60%). In addition, 1H-NMR spectrum of compound 5b exhibited singlet signal at δ3.94 ppm assigned to the protons of two methoxy groups (2OCH₃) for the keto-enol form of compound 5b.
Cytotoxic effect of tested compounds on breast cancer

The cytotoxicity of compounds 4b, 5a, 5b, 6 and 7 was investigated against breast cancer cell line (MCF-7) at concentrations (0.1, 1, 10, and 100 μM) using MTT colorimetric assay. Data illustrated in (Table 2, and Fig. 3) shows the percentage of viability of MCF-7 cells after 48 h from treatment with different concentrations of the compounds versus control.

The ¹H-NMR spectrum of compound 5b displayed multiplet signals in the region of δ7.35–8.09 ppm due to the four protons of two isomers for compound 5b.

The ¹C-NMR spectrum of compound 5 supported the formation of keto-enol form of these compound, because it showed the presented two signals at δ160.51, 160.05 ppm assigned to the carbonyl of amide for the keto form and N= C-OH of enol form. The signals due to the aromatic and C-3, C-4 of pyridine appeared in the region of δ148.50–112.64 ppm of two isomers. Also, the spectrum of compound 5b showed the appearance of two signals of a methoxy group (2OCH₃) for the two isomers at δ56.92 and 56.66 ppm.

The ¹H-NMR spectrum of compound 6 confirmed the absence of any signals corresponding to the protons of NH and OH of keto-enol form, and the proton of OH for carboxylic acid was observed at δ11.10 ppm as a singlet signal. Also, the ¹H-NMR spectrum of compound 6 showed three singlet signals at δ8.53, 3.95 and 2.33 ppm due to the protons of H-4 of quinoline ring, methoxy and acetyl groups, respectively. Protons of the aromatic ring were observed as two doublet signals at δ7.74 and 7.42 ppm.

The ¹C-NMR spectrum of compound 6 showed five signals at δ171.71, 162.31, 158.77, 57.00 and 25.59 ppm assigned to carbon atoms of carbonyl groups of ester and acid, C-2 of quinoline ring, methoxy (OCH₃) and acetyl (COCH₃) groups. The signals due to the quinoline carbon appeared in the region at δ146.77–112.58 ppm.

Biological activity

Cytotoxic effect of tested compounds on liver cancer

The cytotoxicity of each compounds 4b, 5a, 5b, 6 and 7 were investigated against liver cancer cell line (HepG2) at concentrations (0.1, 1, 10, and 100 μM) using MTT colorimetric assay. Data illustrated in (Table 1, and Fig. 2) shows the percentage of viability of HepG2 cells after 48 h from treatment with different concentrations of the compounds versus control.

| Cpd.  | % of cell growth treated with different conc. (μM) of cpds |
|-------|----------------------------------------------------------|
|       | 0.1           | 1             | 10            | 100           |
| 4b    | 66.66667      | 66.66667      | 67.2956       | 60.16771      | 66.87631      | 77.04033 |
| 5a    | 60.16771      | 60.16771      | 62.86344      | 79.66475      | 66.66667      | 39.93711  |
| 5b    | 64.36059      | 64.36059      | 68.46637      | 74.00419      | 90.77568      | 96.01677 |
| 6     | 61.84486      | 61.84486      | 68.70216      | 70.64999      | 69.49686      | 77.04033 |
| 7     | 82.18029      | 82.18029      | 74.42349      | 78.61635      | 72.95597      | 79.24526 |

Table 1. Anticancer activity of compounds 4b, 5a, 5b, 6 and 7 against HepG2 cells

| Cpd.  | % of cell growth treated with different conc. (μM) of cpds |
|-------|----------------------------------------------------------|
|       | 0.1           | 1             | 10            | 100           |
| 4b    | 77.65511      | 76.33718      | 79.76893      | 83.38896      | 93.41891      | 98.74198 |
| 5a    | 87.65083      | 83.67993      | 76.07189      | 80.97561      | 82.29354      | 79.52075 |
| 5b    | 87.73641      | 82.00257      | 81.95122      | 93.68393      | 113.068       | 127.7022 |
| 6     | 83.44031      | 90.44929      | 94.66838      | 92.09675      | 87.92469      | 82.02624 |
| 7     | 91.6303       | 86.55541      | 84.9294       | 95.91784      | 100.4108      | 89.88716 |

Table 2. Anticancer activity of tested compounds against breast cancer (MCF-7 cell line)
which were untreated cell. The IC$_{50}$ (µM) values of 4b, 5a, 5b, 6 and 7 after 48 h continuous exposure of tumor cell lines compared with control give results $>$100 µM leads to inactive except for compound 5a is the most active with IC$_{50}$ value 83.69 µM.

**Table 3.** IC$_{50}$ (µM) values of 4b, 5a, 5b, 6 and 7 after 48 h continuous exposure of tumor cell lines compared with control

| Compound No. | Tumor cell types | MCF-7 | Hep-G2 |
|--------------|------------------|-------|--------|
| 4b           | >100             | >100  |        |
| 5a           | >100             |        | 83.69  |
| 5b           | >100             | >100  |        |
| 6            | >100             | >100  |        |
| 7            | >100             | >100  |        |
| Doxorubicin  |                  | 3.83  | 5.08   |

The cytotoxic effect of compounds 4b, 5a, 5b, 6 and 7 against breast cancer MCF-7 cells: $1 \times 10^4$ cell/well of cells were treated with serial dilution of the tested compounds (0.1, 1, 10, and 100 µM) for 48 h cytotoxic effect was detected by MTT assay. The figure shows the % of cell growth viability compared to control which were untreated cell (n = 3)

**Figure 3.** Cytotoxic effect of the tested compounds against breast cancer MCF-7 cells: $1 \times 10^4$ cell/well of cells were treated with serial dilution of the tested compounds (0.1, 1, 10, and 100 µM) for 48 h cytotoxic effect was detected by MTT assay. The tables (2 & 3) and Fig. 3 shows the % of cell growth viability compared to control which were untreated cell. The IC$_{50}$ (µM) values of 4b, 5a, 5b, 6 and 7 after 48 h continuous exposure of tumor cell lines compared with control give results $>$100 µM which did not record a noticeable effect (inactive) against breast cancer MCF-7 cells.

**CONCLUSION**

A series of hybrid substituted coumarin and azacoumarin-3-carboxylic acid derivatives (4–7) were synthesized and structurally proved using spectral and elemental analysis data. Substituted coumarin-3-carboxylic acid (4a and 5a) and Substituted azacoumarin-3-carboxylic acid (4b, 5b and 6) were tested for their in vitro cytotoxic activity against MCF-7 and HepG-2 cell lines.

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