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SYNTHESIS AND ANTITUMOR ACTIVITY OF 1,3,4-THIADIAZOLE DERIVATIVES BEARING COUMARINE RING

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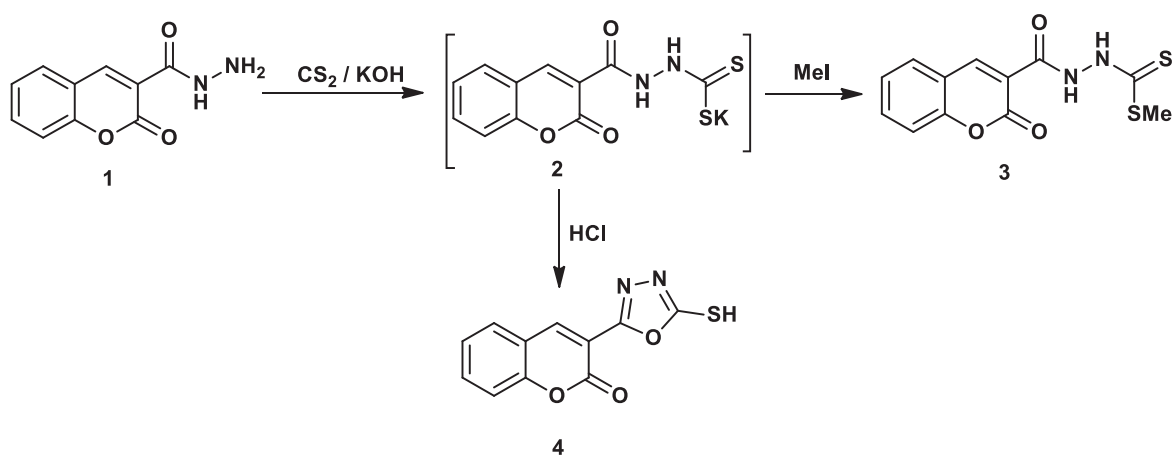
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Abstract – In the present study, preparation of a novel series of *N*-(3,5-diphenyl-1,3,4-thiadiazol-2(3*H*)-ylidene)-2-oxo-2*H*-chromene-3-carbohydrazide (**8a-m**) was prepared by two methods via the reaction of hydrazoneyl halides with methyl 2-(2-oxo-2*H*-chromene-3-carbonyl)hydrazinecarbodithioate or 3-(5-mercapto-1,3,4-oxadiazol-2-yl)-2*H*-chromen-2-one. Structures of the newly synthesized compounds were elucidated on the basis of elemental analyses and spectral data. All the newly synthesized compounds have been evaluated for their antitumor activity against a liver carcinoma cell line HEPG2-1. Also, their structure activity relationship (SAR) was studied. Many of the tested compounds showed moderate to high anticancer activity with respect to doxorubicin as a reference drug.

In continuation of our studies dealing with the utility of hydrazoneyl halides for synthesis of various bridgehead nitrogen polyheterocycles,¹⁻¹⁰ we wish to report herein a new facile synthesis of various functionalized derivatives of 1,3,4-thiadiazoles bearing coumarine moiety that have not been reported hitherto. The synthesis of coumarins and their derivatives have attracted considerable attention from organic and medicinal chemists for many years because of their wide range of medicinal applications such as antitumoral, anti-inflammatory, antiviral, CNS active, anti-HIV, and antioxidant activities.¹¹⁻¹⁶ Moreover, the 1,3,4-thiadiazole nucleus was proven to constitute the active part of several biologically active compounds.¹⁷⁻²¹ In the light of the above findings and in continuation of authors' efforts to

synthesize new anticancer agents,^{5-7, 22-24} we synthesized new series of 1,3,4-thiadiazole derivatives bearing 3-coumarinyl moiety as potential antitumor agents. 2-Oxo-2*H*-chromene-3-carbohydrazide (**1**) was used in the synthesis of potassium 2-(2-oxo-2*H*-chromene-3-carbonyl)hydrazinecarbodithioate **2** as reported in literature.²⁵ Methylation of the salt **2** with MeI in the presence of K₂CO₃ afforded methyl 2-(2-oxo-2*H*-chromene-3-carbonyl)hydrazine-carbodithioate (**3**).²⁶ Acidification of **2** with concentrated HCl yielded 3-(5-mercapto-1,3,4-oxadiazol-2-yl)-2*H*-chromen-2-one (**4**)²⁷ according to the reaction sequence outlined in Scheme 1.



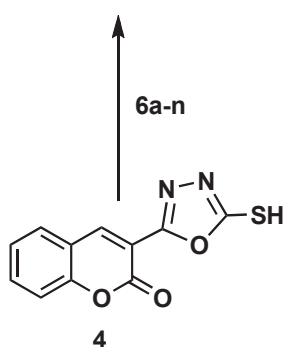
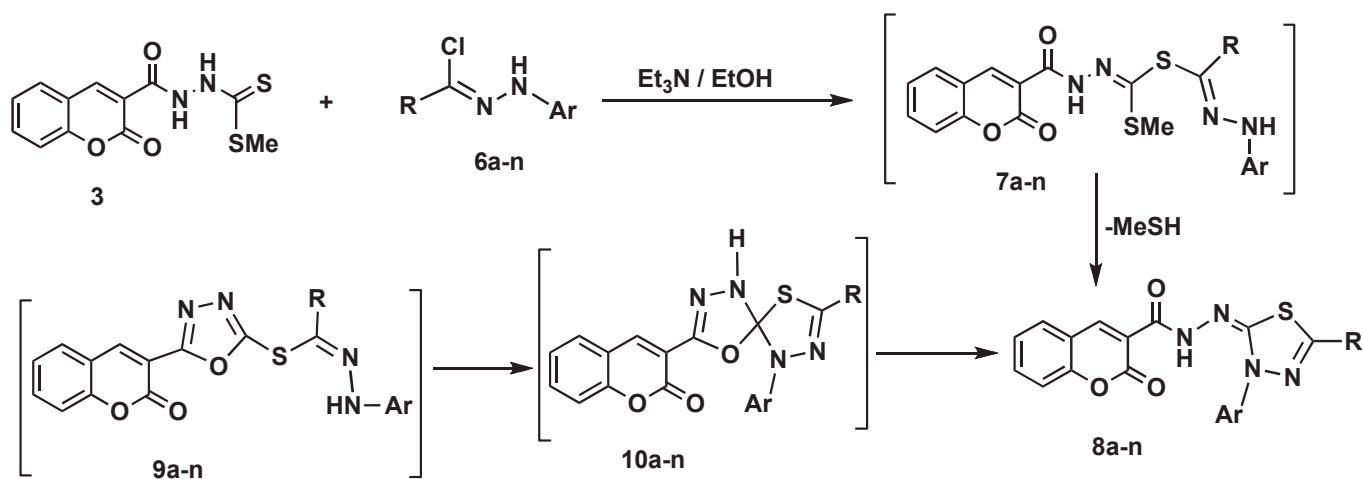
Scheme 1: Synthesis of compounds **3** and **4**

The reaction of methyl 2-(2-oxo-2*H*-chromene-3-carbonyl)hydrazinecarbodithioate (**3**) with hydrazonoyl halides **6a-n** was carried out in ethanol in the presence of triethylamine at room temperature till methyl mercaptan ceased to evolve. The reaction gave in each case after work up, only one isolable product as evidenced by TLC analysis of the crude product. The isolated products were assigned structure **8**, namely *N'*-(3,5-disubstituted-1,3,4-thiadiazol-2(3*H*)-ylidene)-2-oxo-2*H*-chromene-3-carbohydrazide on the basis of elemental analyses and spectral (IR, ¹H NMR, Mass) data. For example, the IR spectra revealed, in each case, the characteristic absorption bands for –CONH– group in the region 3395-3120 and 1676-1642 cm⁻¹. The ¹H NMR spectra exhibited one singlet signal near δ 11.2 ppm which corresponds to the –CONH–proton. The mass spectra revealed, in each case, a peak corresponds to the molecular ion which is consistent with the expected molecular formula. The structure of **8** was proved chemically *via* an alternative method (Scheme 2). Thus, the reaction of compound **4** with **6a-n** in ethanol in the presence of triethylamine at reflux led to formation of product which is identical in all respects (mp, mixed mp and

IR) with compound **8**. The mechanism of formation of compounds **8a-n** is shown in Scheme 2 which is analogous to that reported in literature for related compounds.²⁸⁻³¹ It is assumed that, the final products **8** are formed *via* initial formation of the thiohydrazone esters **9** which underwent *in situ* tandem cyclization followed by ring opening of the 1,3,4-oxadiazole ring. **Antitumor activity**

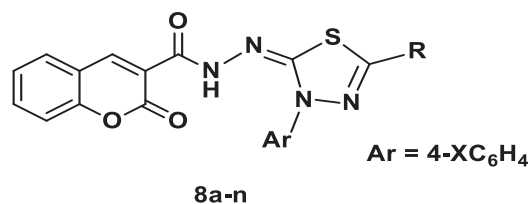
The antitumor activity of the newly synthesized compounds was determined against a liver carcinoma cell line HEPG2-1. Doxorubicin was used as a reference standard and showed $IC_{50} = 0.72 \mu M$ against a liver carcinoma cell line. Data generated were used to plot a dose–response curve of which the concentration (μM) of test compounds required to kill 50% of cell population (IC_{50}) was determined. Cytotoxic activity was expressed as the mean IC_{50} of three independent experiments.

Table 1. Cytotoxic activities of tested compounds against liver carcinoma cell line (HEPG2-1)



Compd. No.	R	Ar	Compd. No.	R	Ar
6a-10a	C ₆ H ₅	C ₆ H ₅	6h-10h	CO ₂ Et	4-MeC ₆ H ₄
6b-10b	Ac	C ₆ H ₅	6i-10i	CO ₂ Et	4-MeOC ₆ H ₄
6c-10c	Ac	4-MeC ₆ H ₄	6j-10j	CO ₂ Et	4-ClC ₆ H ₄
6d-10d	Ac	4-MeOC ₆ H ₄	6k-10k	CONHPh	C ₆ H ₅
6e-10e	Ac	4-ClC ₆ H ₄	6l-10l	CONHPh	4-MeC ₆ H ₄
6f-10f	Ac	4-BrC ₆ H ₄	6m-10m	CONHPh	4-MeOC ₆ H ₄
6g-10g	CO ₂ Et	C ₆ H ₅	6n-10n	CONHPh	4-ClC ₆ H ₄

Scheme 2: Synthesis of thiazole derivatives **8a-m**



Compd No.	R	X	IC ₅₀ (μM)
Doxorubicin	-----	-----	0.72
8j	CO ₂ Et	Cl	0.90
8i	CO ₂ Et	OMe	1.21
8g	CO ₂ Et	H	1.43
8h	CO ₂ Et	Me	1.56
8n	CONHPh	Cl	1.82
8k	CONHPh	H	3.07
8m	CONHPh	OMe	4.78
8l	CONHPh	Me	4.98
8e	Ac	Cl	6.26
8f	Ac	Br	8.25
8d	Ac	OMe	13.43
8b	Ac	H	19.02
8c	Ac	Me	19.25
8a	Ph	H	77.30

The results revealed that most of the tested compounds showed a great variable activity compared to reference drug as shown in Table 1. The descending order of activity of the newly synthesized compounds was as follow: **8j** > **8i** > **8g** > **8h** > **8n** > **8k** > **8m** > **8l** > **8e** > **8f** > **8d** > **8b** > **8c** > **8a**.

Examination of the SAR leads to the following conclusions.

The 1,3,4-thiadiazole derivatives **8j**, **8i**, **8g**, **8h** and **8n** (IC₅₀ = 0.90, 1.21, 1.43, 1.56, and 1.82 μM, respectively) have promising antitumor activity against liver carcinoma cell line (HEPG2-1) while 1,3,4-thiadiazole derivatives **8k**, **8m**, **8l**, **8e**, **8f**, **8d**, **8b** and **8c** have moderate activity (IC₅₀ = 3.07 -19.25 μM). On the other hand, 1,3,4-thiadiazole derivative **8a** has poor antitumor activity against liver carcinoma cell line (IC₅₀ = 77.3 μM).

- For substituent at position 2 of 1,3,4-thiadiazole: the ester group (CO₂Et) give higher activity than the amide group (CONHPh) than the acetyl group (Ac) than the phenyl moiety (Ph).

CONCLUSION

In summary, efficient syntheses and characterization of new 1,3,4-thiadiazole system by two routes have been reported. The structure of all the newly synthesized compounds was established by elemental and

spectral analyses. All the synthesized compounds were evaluated for their anti-cancer activity against the liver carcinoma cell line. Also, their structure activity relationship (SAR) was studied. The results revealed that 1,3,4-thiadiazole derivatives **8i** and **8j** have promising antitumor activities ($IC_{50} = 1.21$ and $0.90 \mu\text{M}$, respectively) against liver carcinoma cell line and most of the tested compounds showed moderate anti-cancer activities.

EXPERIMENTAL

Melting points were measured on an Electrothermal IA 9000 series digital melting point apparatus. IR spectra were recorded in potassium bromide discs on Pye Unicam SP 3300 and Shimadzu FTIR 8101 PC infrared spectrophotometers. NMR spectra were recorded on a Varian Mercury VX-300 NMR spectrometer operating at 300 MHz (^1H NMR) or 75 MHz (^{13}C NMR) and run in deuterated dimethyl sulfoxide ($\text{DMSO-}d_6$). Chemical shifts were related to that of the solvent. Mass spectra were recorded on a Shimadzu GCeMS-QP1000 EX mass spectrometer at 70 eV. Elemental analyzes were measured by using a German made Elementar vario LIII CHNS analyzer. Antitumor activity was evaluated by the Regional Center for Mycology and Biotechnology, Al-Azhar University, Cairo, Egypt. Hydrazonoyl chloride³²⁻³⁴ was prepared as previously reported in the respective literature.

Synthesis of *N'*-(5-substituted-3-aryl-1,3,4-thiadiazol-2(3*H*)-ylidene)-2-oxo-2*H*-chromene-3-carbohydrazide (**8a-m**)

Method A: To a mixture of methyl 2-(2-oxo-2*H*-chromene-3-carbonyl)hydrazinecarbodithioate (**3**) (0.294 g, 1 mmol) and the appropriate hydrazonoyl chloride **6a-m** (1 mmol) in absolute EtOH (20 mL), was added triethylamine (0.07 mL, 1 mmol). The reaction mixture was stirred at room temperature till methyl mercaptan ceased to evolve (2 h). The solvent was evaporated and the residue was treated with ice/HCl mixture. The solid product was collected, washed with EtOH, dried, and finally recrystallized from the appropriate solvent to give the respective products **8a-m**.

Method B: A mixture of the oxadiazole derivative **4** (0.246 g, 1 mmol) and hydrazonoyl chlorides **6a-m** (1 mmol) in absolute EtOH (20 mL) was refluxed for 6 h. The solvent was then evaporated and the solid left was collected, washed with water, dried and finally recrystallized from EtOH to give product proved to be identical in all respects (mp, mixed mp and IR spectra) with the product **8** which obtained from reaction of **3** with **6** but in lower yield%.

N'-(3,5-Diphenyl-1,3,4-thiadiazol-2(3*H*)-ylidene)-2-oxo-2*H*-chromene-3-carbohydrazide

(8a). Yellow solid; 88% yield; mp 212-214 °C; IR (KBr): ν 3312 (NH), 1714, 1678 (2C=O) cm^{-1} ; ^1H NMR (DMSO- d_6): δ 6.78-7.88 (m, 14H, ArH), 8.30 (s, 1H, Coumarine-H4), 10.24 (s, br, 1H, NH); ^{13}C NMR (DMSO- d_6): δ 118.2, 120.5, 121.2, 122.6, 123.0, 125.4, 127.4, 130.3, 130.7, 132.7, 136.9, 137.2, 140.1, 141.6, 142.2, 145.6, 148.6, 158.3 (Ar-C), 163.3 (C=O), 169.2 (C=O); MS m/z (%): 441 ($\text{M}^+ + 1$, 21), 440 (M^+ , 10), 264 (30), 158 (100), 105 (80). Anal. Calcd for $\text{C}_{24}\text{H}_{16}\text{N}_4\text{O}_3\text{S}$ (440.47): C, 65.44; H, 3.66; N, 12.72. Found C, 65.28; H, 3.54; N, 12.60%.

***N'*-(5-Acetyl-3-phenyl-1,3,4-thiadiazol-2(3*H*)-ylidene)-2-oxo-2*H*-chromene-3-carbohydrazide**

(8b). Yellow solid; 84% yield; mp 254-256 °C; IR (KBr): ν 3343 (NH), 1713, 1690, 1672 (3C=O) cm^{-1} ; ^1H NMR (DMSO- d_6): δ 2.44 (s, 3H, CH_3), 6.70-7.86 (m, 9H, ArH), 8.32 (s, 1H, Coumarine-H4), 10.41 (s, br, 1H, NH); ^{13}C NMR (DMSO- d_6): δ 22.3, 118.6, 120.4, 121.9, 123.7, 124.6, 125.9, 128.6, 130.2, 133.9, 136.2, 139.2, 140.3, 142.3, 149.4 (Ar-H), 163.3, 167.3, 191.6 (C=O); MS m/z (%): 406 (M^+ , 13), 377 (54), 156 (100), 77 (59). Anal. Calcd for $\text{C}_{20}\text{H}_{14}\text{N}_4\text{O}_4\text{S}$ (406.41): C, 59.11; H, 3.47; N, 13.79. Found C, 59.01; H, 3.27; N, 13.55%.

***N'*-(5-Acetyl-3-*p*-tolyl-1,3,4-thiadiazol-2(3*H*)-ylidene)-2-oxo-2*H*-chromene-3-carbohydrazide**

(8c). Yellow solid; 86% yield; mp 212-214 °C; IR (KBr): ν 3318 (NH), 1713, 1686, 1670 (3C=O) cm^{-1} ; ^1H NMR (DMSO- d_6): δ 2.32 (s, 3H, CH_3), 2.41 (s, 3H, CH_3), 6.71-7.77 (m, 8H, ArH), 8.27 (s, 1H, Coumarine-H4), 10.34 (s, br, 1H, NH); MS m/z (%): 420 (M^+ , 8), 254 (14), 158 (100), 77 (63). Anal. Calcd for $\text{C}_{21}\text{H}_{16}\text{N}_4\text{O}_4\text{S}$ (420.44): C, 59.99; H, 3.84; N, 13.33. Found C, 59.78; H, 3.65; N, 13.19%.

***N'*-(5-Acetyl-3-(4-methoxyphenyl)-1,3,4-thiadiazol-2(3*H*)-ylidene)-2-oxo-2*H*-chromene-3-**

carbohydrazide (8d). Yellow solid; 87% yield; mp 178-180 °C; IR (KBr): ν 3320 (NH), 1709, 1688, 1675 (3C=O) cm^{-1} ; ^1H NMR (DMSO- d_6): δ 2.41 (s, 3H, CH_3), 3.58 (s, 3H, CH_3), 6.64-7.82 (m, 8H, ArH), 8.35 (s, 1H, Coumarine-H4), 10.40 (s, br, 1H, NH); MS m/z (%): 436 (M^+ , 19), 320 (61), 250 (29), 158 (100). Anal. Calcd for $\text{C}_{21}\text{H}_{16}\text{N}_4\text{O}_5\text{S}$ (436.44): C, 57.79; H, 3.70; N, 12.84. Found C, 57.65; H, 3.52; N, 12.57%.

***N'*-(5-Acetyl-3-(4-chlorophenyl)-1,3,4-thiadiazol-2(3*H*)-ylidene)-2-oxo-2*H*-chromene-3-**

carbohydrazide (8e). Yellow solid; 89% yield; mp 212-214 °C; IR (KBr): ν 3322, 3129 (2NH), 1712, 1688, 1673 (3C=O) cm^{-1} ; ^1H NMR (DMSO- d_6): δ 2.43 (s, 3H, CH_3), 6.68-7.87 (m, 13H, ArH), 8.33 (s, 1H, Coumarine-H4), 10.42 (s, br, 1H, NH); MS m/z (%): 442 ($\text{M}^+ + 2$, 4), 440 (M^+ , 15), 310 (43), 186 (73), 105 (47), 77 (100). Anal. Calcd for $\text{C}_{20}\text{H}_{13}\text{ClN}_4\text{O}_4\text{S}$ (440.86): C, 54.49; H, 2.97; N, 12.71. Found C, 54.40; H, 2.88; N, 12.65%.

***N'*-(5-Acetyl-3-(4-bromophenyl)-1,3,4-thiadiazol-2(3*H*)-ylidene)-2-oxo-2*H*-chromene-3-carbohydrazone (8f).** Yellow solid; 86% yield; mp 235-237 °C; IR (KBr): ν 3343 (NH), 1712, 1686, 1670 (3C=O) cm^{-1} ; ^1H NMR (DMSO- d_6): δ 2.42 (s, 3H, CH₃), 6.65-7.89 (m, 8H, ArH), 8.37 (s, 1H, Coumarine-H4), 10.41 (s, br, 1H, NH); MS m/z (%): 487 (M⁺ + 2, 14), 578 (M⁺, 16), 423 (26), 355 (24), 245 (32), 205 (34), 149 (45), 69 (100). Anal. Calcd for C₂₀H₁₃BrN₄O₄S (485.31): C, 49.50; H, 2.70; N, 11.54. Found C, 49.39; H, 2.62; N, 11.35%.

Ethyl 5-(2-(2-oxo-2*H*-chromene-3-carbonyl)hydrazono)-4-phenyl-4,5-dihydro-1,3,4-thiadiazole-2-carboxylate (8g). Yellow solid; 84% yield; mp 170-172 °C; IR (KBr): ν 3322 (NH), 1743, 1688, 1667 (3C=O) cm^{-1} ; ^1H NMR (DMSO- d_6): δ 1.36 (t, 3H, $J = 7.2$ Hz, CH₂CH₃), 4.24 (q, 2H, $J = 7.2$ Hz, CH₂CH₃), 6.69-7.87 (m, 9H, ArH), 8.33 (s, 1H, Coumarine-H4), 10.42 (s, br, 1H, NH); ^{13}C NMR (DMSO- d_6): δ 16.4, 53.8, 117.6, 120.2, 121.6, 122.6, 125.7, 130.6, 130.8, 132.0, 136.7, 138.5, 140.1, 141.6, 147.9, 156.5 (Ar-C), 161.8, 163.5, 169.7 (C=O); MS m/z (%): 436 (M⁺, 19), 284 (9), 158 (100), 130 (34), 95 (68). Anal. Calcd for C₂₁H₁₆N₄O₅S (436.44): C, 57.79; H, 3.70; N, 12.84. Found C, 57.71; H, 3.65; N, 12.66%.

Ethyl 5-(2-(2-oxo-2*H*-chromene-3-carbonyl)hydrazono)-4-*p*-tolyl-4,5-dihydro-1,3,4-thiadiazole-2-carboxylate (8h). Yellow solid; 83% yield; mp 183-185 °C; IR (KBr): ν 3322 (NH), 1744, 1683, 1668 (3C=O) cm^{-1} ; ^1H NMR (DMSO- d_6): δ 1.38 (t, 3H, $J = 7.2$ Hz, CH₂CH₃), 2.43 (s, 3H, CH₃), 4.28 (q, 2H, $J = 7.2$ Hz, CH₂CH₃), 6.68-7.87 (m, 8H, ArH), 8.33 (s, 1H, Coumarine-H4), 10.42 (s, br, 1H, NH); MS m/z (%): 450 (M⁺, 19), 254 (50), 155 (100), 77 (60). Anal. Calcd for C₂₂H₁₈N₄O₅S (450.47): C, 58.66; H, 4.03; N, 12.44. Found C, 58.48; H, 4.01; N, 12.35%.

Ethyl 4-(4-methoxyphenyl)-5-(2-(2-oxo-2*H*-chromene-3-carbonyl)hydrazono)-4,5-dihydro-1,3,4-thiadiazole-2-carboxylate (8i). Yellow solid; 80% yield; mp 171-173 °C; IR (KBr): ν 3322 (NH), 1740, 1678, 1660 (3C=O) cm^{-1} ; ^1H NMR (DMSO- d_6): δ 1.38 (t, 3H, $J = 7.2$ Hz, CH₂CH₃), 3.78 (s, 3H, CH₃), 4.32 (q, 2H, $J = 7.2$ Hz, CH₂CH₃), 6.68-7.87 (m, 8H, ArH), 8.33 (s, 1H, Coumarine-H4), 10.42 (s, br, 1H, NH); MS m/z (%): 466 (M⁺, 19), 316 (100), 105 (76). Anal. Calcd for C₂₂H₁₈N₄O₆S (466.47): C, 56.65; H, 3.89; N, 12.01. Found C, 56.48; H, 3.80; N, 11.92%.

Ethyl 4-(4-chlorophenyl)-5-(2-(2-oxo-2*H*-chromene-3-carbonyl)hydrazono)-4,5-dihydro-1,3,4-thiadiazole-2-carboxylate (8j). Yellow solid; 83% yield; mp 193-195 °C; IR (KBr): ν 3322 (NH), 1749, 1684, 1658 (3C=O) cm^{-1} ; ^1H NMR (DMSO- d_6): δ 1.38 (t, 3H, $J = 7.2$ Hz, CH₂CH₃), 4.28 (q, 2H, $J = 7.2$ Hz, CH₂CH₃), 6.68-7.87 (m, 8H, ArH), 8.33 (s, 1H, Coumarine-H4), 10.42 (s, br, 1H, NH); MS m/z (%):

470 (M^+ , 19), 158 (40), 105 (100). Anal. Calcd for $C_{21}H_{15}ClN_4O_5S$ (470.89): C, 53.56; H, 3.21; N, 11.90. Found C, 53.63; H, 3.08; N, 11.69%.

5-(2-(2-Oxo-2H-chromene-3-carbonyl)hydrazono)-N,4-diphenyl-4,5-dihydro-1,3,4-thiadiazole-2-carboxamide (8k). Yellow solid; 89% yield; mp 207-209 °C; IR (KBr): ν 3322, 3129 (2NH), 1688, 1673, 1648 (3C=O) cm^{-1} ; 1H NMR (DMSO- d_6): δ 6.68-7.87 (m, 14H, ArH), 8.33 (s, 1H, Coumarine-H4), 10.45 (s, br, 1H, NH), 11.31 (s, br, 1H, NH); MS m/z (%): 511 (M^+ , 19), 282 (14), 105 (100), 158 (76). Anal. Calcd for $C_{25}H_{17}N_5O_4S$ (511.51): C, 62.10; H, 3.54; N, 14.48. Found C, 62.02; H, 3.27; N, 14.42%.

5-(2-(2-Oxo-2H-chromene-3-carbonyl)hydrazono)-N-phenyl-4-(p-tolyl)-4,5-dihydro-1,3,4-thiadiazole-2-carboxamide (8l). Yellow solid; 88% yield; mp 188-190 °C; IR (KBr): ν 3335, 3163 (2NH), 1689, 1672, 1646 (3C=O) cm^{-1} ; 1H NMR (DMSO- d_6): δ 2.30 (s, 3H, CH_3), 6.68-7.87 (m, 13H, ArH), 8.33 (s, 1H, Coumarine-H4), 10.45 (s, br, 1H, NH), 11.31 (s, br, 1H, NH); MS m/z (%): 497 (M^+ , 19), (100), 130 (36), 103 (16), 77 (18). Anal. Calcd for $C_{26}H_{19}N_5O_4S$ (497.53): C, 62.77; H, 3.85; N, 14.08. Found C, 62.79; H, 3.71; N, 13.87%.

4-(4-Chlorophenyl)-5-(2-(2-oxo-2H-chromene-3-carbonyl)hydrazono)-N-phenyl-4,5-dihydro-1,3,4-thiadiazole-2-carboxamide (8m). Yellow solid; 88% yield; mp 223-225 °C; IR (KBr): ν 3322, 3129 (2NH), 1687, 1670, 1653 (3C=O) cm^{-1} ; 1H NMR (DMSO- d_6): δ 6.68-7.87 (m, 13H, ArH), 8.33 (s, 1H, Coumarine-H4), 10.45 (s, br, 1H, NH), 11.31 (s, br, 1H, NH); MS m/z (%): 517 (M^+ , 19), 254 (70), 158 (100), 77 (22). Anal. Calcd for $C_{25}H_{16}ClN_5O_4S$ (517.94): C, 57.97; H, 3.11; N, 13.52. Found C, 57.74; H, 3.18; N, 13.38%.

Evaluation of the antitumor activity using Viability assay:

Human hepatocellular carcinoma (HEPG2) cell line was obtained from the American Type Culture Collection (ATCC, Rockville, MD). The cells were grown on RPMI-1640 medium supplemented with 10% inactivated fetal calf serum and 50 $\mu g/mL$ gentamycin. The cells were maintained at 37 °C in a humidified atmosphere with 5% CO_2 and were subcultured two to three times a week. Potential cytotoxicity of the compounds was evaluated on tumor cells using the method of Gangadevi and Muthumary.³⁵ The cells were grown as monolayers in growth RPMI-1640. The monolayers of 10^4 cells adhered at the bottom of the wells in a 96-well microtiter plate incubated for 24 h at 37 °C in a humidified incubator with 5% CO_2 . The monolayers were then washed with sterile phosphate buffered saline (0.01 M pH 7.2) and simultaneously the cells were treated with 100 μL from different dilutions of tested sample in fresh maintenance medium and incubated at 37 °C. A control of untreated cells was made in the absence

of tested sample. Positive controls containing doxorubicin drug was also tested as reference drug for comparison. Six wells were used for each concentration of the test sample. Every 24 h the observation under the inverted microscope was made. The number of the surviving cells was determined by staining the cells with crystal violet³⁶ followed by cell lysing using 33% glacial acetic acid and read the absorbance at 590 nm using microplate reader (SunRise, TECAN, Inc, USA) after well mixing. The absorbance values from untreated cells were considered as 100% proliferation. The number of viable cells was determined using microplate reader as previously mentioned before and the percentage of viability was calculated as $[1-(OD_t/OD_c)] \times 100\%$ where OD_t is the mean optical density of wells treated with the tested sample and OD_c is the mean optical density of untreated cells. The relation between surviving cells and drug concentration is plotted to get the survival curve of each tumor cell line after treatment with the specified compound. The 50% inhibitory concentration (IC_{50}), the concentration required to cause toxic effects in 50% of intact cells, was estimated from graphic plots.

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