Selectivity of *N*-Aroyl-*N*′-arylthioureas towards 2-(1,3-Dioxo-1*H*-inden-2(3*H*)-ylidene)malononitrile. New Synthesis of (*Z*)-*N*-((*E*)-4-Amino-1-aryl-5-cyano-6-oxo-1*H*-indeno[1,2-*d*][1,3]-thiazepin-2(6*H*)-ylidene)-4-arylamides of Antitumor and Antioxidant Activities

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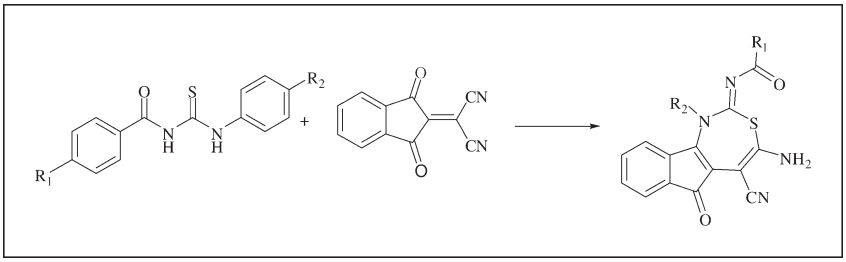

The reaction between *N*-aroyl-*N*′-arylthioureas with 2-(1,3-dioxoindan-2-ylidene)malononitrile furnished indeno[1,2-*d*][1,3]thiazepines in 70–85% yields. The mechanism of the products' formation is discussed. Some of the products showed effective antitumor and antioxidant activities. The results revealed that compound indenothiazepine derivative showed a high inhibition of the cell growth of Hep-G2 cells is compared with the growth of untreated control cells, as concluded from their low IC_{50} value 21.73 μ*M*. On the other hand, two indenothiazepine derivatives have an effective antioxidant activity with SC_{50} values of 62.5 m*M* and 87.4 m*M*, respectively.

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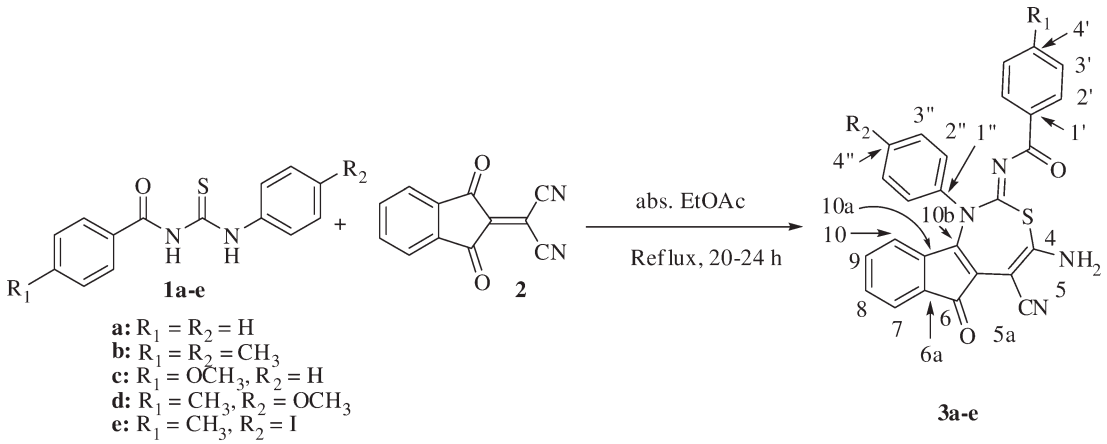
INTRODUCTION

The importance of *N*-aroyl-*N*′-arylthioureas is found largely in heterocyclic syntheses. Many of these substrates have interesting biological activities and are used as a rich source for materials for development of agrochemical and pharmaceutical products [1]. Additionally, it was known that the reaction of amidinothioureas, imidoylthioureas, thioacylamidines, amidino-thioureas, *o*-methyl-1-aryl-2-thioisobiurets, and 1-aryl-isodithiobiurets with diethyl azodicarboxylate gave the corresponding thiadiazoles by the oxidative cyclic S—N bond formation [2]. A series of 3-alkyl-5-methylene-2-arylimino-1,3-thiazolidin-4-ones were obtained from the reaction of *N*-alkyl-*N*′-arylthioureas with dimethyl but-2-ynedioate [3]. Hyrazino-thioureas, such as 1-acylthiosemicarbazides reacted with phenyl propiolate in acetic acid under reflux to afford triazolothiazines [3]. In light of the aforementioned, it appears that the reaction pathways of substituted thioureas vary from one reagent to another. Our synthetic program uses cycloadditions as efficient methods of preparation of novel heterocycles, rather than those suffering from low yields because of the multiple steps described in their preparation [4]. Aly *et al*. [5] reported the synthesis of a series of 1,3-thiazines by the reaction of *N*-aroyl-*N*′-substituted thioureas with ethyl propiolate, dimethyl but-2-ynedioate, and (*E*)-1,4-diphenyl-but-2-ene-1,4-dione. Herein we report on our findings for the synthesis of various novel thiazepines, during the reaction of various *N*-aroyl-*N*′-aryl-thioureas **1a–e** [6] with 2-(1,3-dioxo-1*H*-inden-2(3*H*)-ylidene)-malononitrile (**2**).

RESULTS AND DISCUSSION

Chemistry. Scheme 1 outlines the reaction of **1a–e** with **2** in dry ethyl acetate under N_2 atmosphere. The

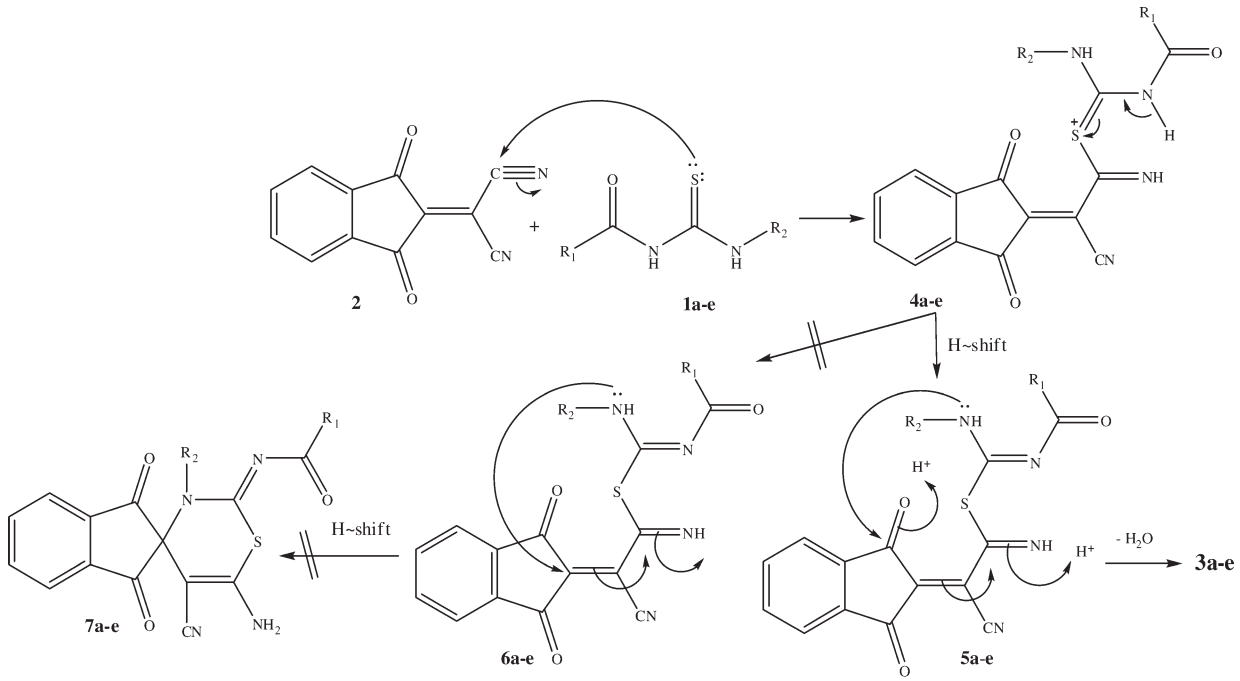
Scheme 1. Reactions of aroyl thioureas **1a–e** with **2**; synthesis of (*Z*)-*N*-((*E*)-4-amino-1-aryl-5-cyano-6-oxo-1*H*-indeno[1,2-*d*][1,3]thiazepin-2(6*H*)-ylidene)-4-arylamides **3a–e**. **3a**: 75%; **3b**: 80%; **3c**: 85%; **3d**: 82%; **3e**: 70%.



reaction proceeded to yield, after chromatographic purification and recrystallization, compounds **3a–e** (70–85%). We chose *N*-aroyl-*N*′-arylthioureas **1a–e** having aryl groups with electron donating and withdrawing substituents on the benzene ring to examine their effect on the course of reaction. The structure of **3a–e** was established on the basis of mass, IR, ^{1}H NMR, ^{13}C NMR spectra, and elemental analyses. Mechanistically, the formation of compounds **3a–e** can be explained as because of addition of the sulfur atom nonbonded pair of **1a–e** to the nitrile group in **2** (Scheme 2). The formed intermediate **4** undergoes a hydrogen shift to give **5**. Cyclization then occurs *via* addition of the NH lone-pair to the carbonyl group, followed by elimination of water, to give the stable heterocyclic compounds **3a–e**. The NMR spectra excluded the formation of *spiro*-indolothiazines **7a–e**, but all the data are consistent with indeno[1,2-*d*][1,3]thiazepines **3a–e** (Scheme 2).

Compounds **3a–e** show IR absorptions at $\nu = 3120–3350$, 2222–2200, 1733–1665, and 1649–1595 cm^{-1} corresponding to the NH_2, nitrile, carbonyl, and azomethine groups, respectively. The spectra are strikingly similar: clearly the products are of the same general type, and share some substructures. The spectra show

Scheme 2. Suggested mechanism of the reaction of **1a–e** with **2**.



local symmetry only in the aryl rings, which can apparently rotate about their axes; the indanedione-derived substructures show individual signals for each proton and each carbon, requiring these substructures to lack the plane of symmetry present in **2** and the alternative products **7a–e**. Each ^{1}H NMR spectrum showed a broad, 2H signal near $\delta_H = 8.20$ ppm, which gives no heteronuclear multiple quantum coherence (HMQC) or heteronuclear single quantum coherence (HSQC) correlation, suggesting that these protons are not attached to carbon. In **3e**, the *p*-tolyl C—CH_3 group is distinctive at $\delta_H = 2.31$ and $\delta_C = 21.2$ ppm. The proton signal at $\delta_H = 2.31$ ppm gives heteronuclear multiple bond correlation correlation (HMBC) correlation with one of the signals at $\delta_C = 143.3$ ppm, which is assigned as C-4′. The C—CH_3 ^{1}H signal gives COSY correlation with $\delta_H = 7.21$ ppm, and C-4′ gives HMBC correlation with $\delta_H = 7.69$ ppm; these correlations suggest that $\delta_H = 7.21$ and 7.69 ppm are H-3′ and H-2′, respectively. These signals showed HMQC correlation with $\delta_C = 129.0$ and 129.3 ppm, respectively. The *p*-toluamide carbonyl at $\delta_C = 175.2$ ppm gives HMBC with $\delta_H = 7.69$ but not 7.21 ppm, which is consistent with the foregoing. The spectra of **3b** contain signals identical to those just described within $\delta_H = 0.02$, $\delta_C = 0.07$ ppm, and $J = 0.1$ Hz; these signals are assigned to the toluamide substructure of **3b**. In the HMBC spectrum of **3b**, correlation is observed between H-3′ and $\delta_C = 132.6$ not 133.3 ppm; therefore, the former is assigned as C-1′. By analogy with the chemical shifts of **3e**, $\delta_C = 132.6$ is assigned as C-1′, and $\delta_C = 143.3$ ppm is assigned as C-4′. Similarly, in **3c** the methoxy group is distinctive at $\delta_H = 3.77$ and $\delta_C = 55.4$ ppm. This proton signal gives HMBC correlation with a signal at $\delta_C = 163.0$ ppm, which is assigned as C-4′. C-4′ gives HMBC correlation with proton signals at $\delta_H = 7.75$ and 6.91 ppm, which thus must be H-2′ and H-3′. The amide carbonyl at $\delta_C = 174.6$ gives HMBC correlation with $\delta_H = 7.75$ ppm, which leads to assignment of this proton signal as H-2′. By elimination, $\delta_H = 6.91$ ppm must be H-3′. H-2′ and H-3′ give HSQC correlation with $\delta_C = 132.6$ and 113.7 ppm, which therefore are assigned as C-2′ and C-3′, respectively. H-3′ gives HMBC correlation with the nonprotonated carbon at $\delta_C = 127.4$ ppm, which is assigned as C-1′. The signals at $\delta_C = 115.0$–116.0 ppm are assigned as nitrile carbons [7]. The carbons at $\delta_C = 136.6$, 132.2, 125.5, and 125.0 give HMQC correlation with proton signals at $\delta_H = 7.76$, 7.76, 7.98, and 6.69 ppm, again with little variation in the chemical shifts.

The most distinctive of these proton signals is that at $\delta_H = 6.69$ ppm, which is a doublet in **3b** and **3c**, suggesting that it is one of the end protons of the four-spin system (either H-7 or H-10). For the moment arbitrarily assigning this proton as H-10, the attached carbon (HMQC: $\delta_C = 125.0$ ppm) is assigned as C-10. H-10 also gives COSY correlation with one of the two unresolved protons at $\delta_H = 7.76$ ppm, which therefore is H-8, 9. These protons give HMQC correlation with the carbons at $\delta_C = 136.6$ and 132.2 ppm. The latter carbons give HMBC correlation to H-10; the former does not. C-9 is two bonds from H-10, C-8 is three bonds away; as three-bond C—H couplings usually give stronger HMBC correlations than two-bond couplings, $\delta_C = 132.2$ ppm is assigned as C-8 and $\delta_C = 136.6$ ppm is assigned as C-9. C-9 gives HMBC correlation with the signal at $\delta_H = 7.98$ ppm, which thus is assigned as H-7 and its attached carbon ($\delta_C = 125.5$ ppm) as C-7. In **3b**, H-7 appears as a double of doublet with large and small coupling constants ($J = 6.6$, 1.4 Hz), as expected in this position. The remaining ^{13}C signals are those at $\delta_C = 193.2$, 170.2, 165.9, 143.4, 133.4, 105.0, 70.9, and 53.1 ppm. The remaining carbon atoms are C-6, 2, 4, 10a, 6a, 10b, 5a. and 5; they are assigned in the order stated. In **3b** and **3c**, the signal at $\delta_C = 105.2$ ppm gives HMBC correlation to H-10, so it was assigned as C-10b. Also in **3b** and **3c**, the signal at $\delta_C = 143.5$ ppm gives HMBC correlation to H-7 and either H-8 or H-9; this is expected for C-10a, as which this signal is therefore assigned. In **3e** and **3b**, the signal at $\delta_C = 53.2$ ppm gives HMBC correlation to the remaining proton signal at $\delta_H = 8.22$ ppm, which has an integral of 2H, and gives no HMQC correlation in any of the compounds; its only other HMBC correlation is in **3b** to $\delta_C = 165.9$. This idea is consistent with chemical-shift simulation using ChemNMR, which predicts for **3b** that C4, 5, and 5a will resonate at $\delta_C = 152$, 9, and 107.0 ppm, respectively.

The most surprising thing here is the predicted chemical shift of C-5, which is unusually far upfield for an sp^2-hybridized carbon. The rationale would be that the electron density at C-5 is higher than normal, due to resonance donation by the nitrogen on C-4. However, the assigned experimental shifts are very close to those of 1,1-dimethoxyethene, and enamines behave similarly to enol ethers (Fig. 1). The δ_C values for C-4 and C-5 are in accordance with the observed trends in the δ_C values in push–pull system in alkenes [8]. It is consistent with these assignments, however, that the amine protons give HMBC correlation to C-4 and C-5. The remaining upfield signal at $\delta_C = 70.9$ ppm is assigned as C-5a.

Biological section.

Anticancer activity. The cytotoxicity testing of compounds **3b–d** was carried out using solid tumor (Hep-G2) cells, which were treated with different doses of the tested compounds and submitted to MTT assay [9]. The yellow tetrazolium salt is reduced by mitochondrial enzyme succinate dehydrogenase, present in living cells, to form insoluble purple formazan crystals, which are solubilized by the addition of detergent. The relative viable cells were determined by the amount of MTT

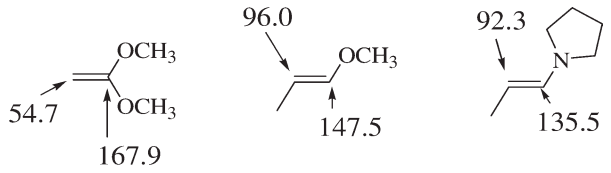


Figure 1. Assigned chemical shift values of the carbon signals in 1,1-dimethoxyethene and enamines.

converted to the insoluble formazan crystals. The data were expressed as the mean percentage of the viable cells as compared with the respective control cultures treated with solvent. Half-maximal growth inhibitory concentration (IC_{50}) values were calculated from the line equation of the dose-dependent curve of each compound. Compound **3b** resulted in a high inhibition of the cell growth of Hep-G2 cells compared with the growth of untreated control cells, as concluded from the IC_{50} value of 21.73 μ*M*. on the other hand, compounds **3c**,**d** led to insignificant change in the growth of Hep-G2 cells as indicated from their IC_{50} values (>100 μ*M*). Results are represented as percentage of control untreated cells as shown in Figures 2–4.

Antioxidant activity. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) is a stable nonphysiological radical, which could provide a relative figure of the radical scavenging activity of the tested compounds [10]. The DPPH assay showed that **3c** possessed no scavenging activity to DPPH with high SC_{50} values (>100 μ*M*) compared with the scavenging activity (SC_{50} 8.41) of the well-known antioxidant (ascorbic acid); on the other hand, compounds **3b** and **3d** had effective antioxidant activity with SC_{50} values of 62.5 and 87.4 μ*M*, respectively (Fig. 5).

EXPERIMENTAL

Chemistry: General methods. *N*-Aroyl-*N*′-arylthioureas **1a–e** were prepared according to literature [6], whereas 2-(1,3-dioxoindan-2-ylidene)-malononitrile (**2**) was prepared according to literature [8]. IR spectra were measured of KBr pellets; absorption frequencies (ν) are stated in cm^{-1}. NMR spectra were measured in DMSO-d_6 solution, at 400.13 MHz for ^1H and 100.6 MHz for ^{13}C; chemical shifts are stated in ppm (δ), and coupling constants are stated in Hz.

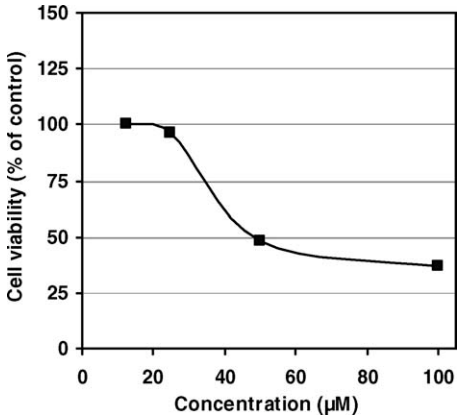


Figure 3. The effect of compound **3c** on the growth of Hep-G2 cells. As measured by MTT assay. As measured by MTT assay. Results are represented as percentage of control untreated cells.

Chemistry

Reaction between N-aroyl-N′-arylthioureas 1a–e and 2-(1,3-dioxo-1H-inden-2(3H)-ylidene)malononitrile (CNIND, 2). To a solution of **2** (0.208 g, 1 mmol) in dry ethyl acetate (10 mL) a solution of **1a–e** (1 mmol) in dry ethyl acetate (10 mL) was added over 10 min at room temperature with stirring. The reaction mixture was continued with stirring at refluxing temperature for 24–20 h. The reaction mixture was concentrated and the residue was separated by preparative TLC (silica gel) using toluene:ethyl acetate (2:1) as eluant. The major zones were extracted with acetone. The isolated products **3a–e** were recrystallized from the stated solvents.

(Z)-N-(E)-4-Amino-5-cyano-6-oxo-1-phenyl-1H-indeno[1,2-d][1,3]thiazepin-2(6H)-ylidene)benzamide (3a). Yellowish white crystals (DMF/H_2O, 10:1), 336 mg (75%), m.p. 257–259°C. IR: 3284, 3123 (m, NH_2), 3048–3000 (m, Ar-CH), 2989–2913 (m, aliph.-CH), 2213 (s, CN), 1729, 1667 (s,

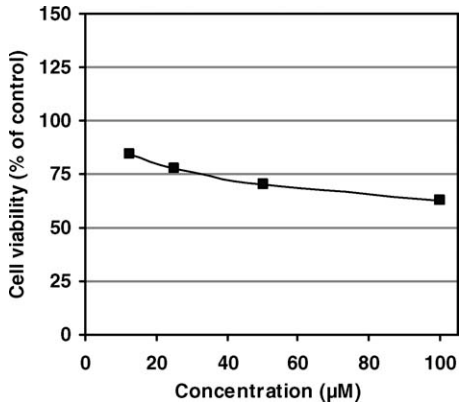


Figure 2. The effect of compound **3b** on the growth of Hep-G2 cells. As measured by MTT assay. Results are represented as percentage of control untreated cells.

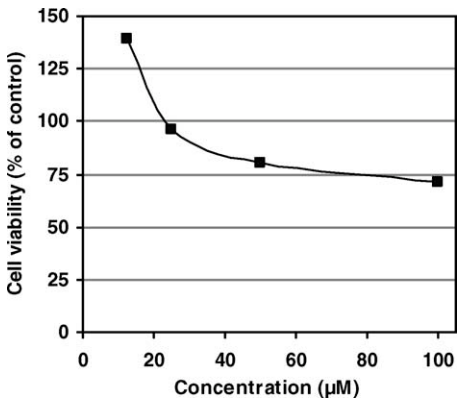


Figure 4. The effect of **3d** on the growth of Hep-G2 cells. As measured by MTT assay. Results are represented as percentage of control untreated cells.

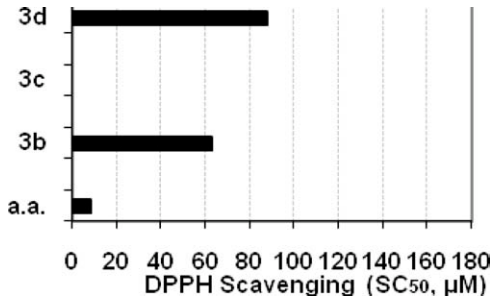


Figure 5. The antioxidant activity of **3b**, **3c,** and **3d** was investigated using DPPH assay. The results are represented as SC_{50} values (μM) as (Mean± S.E, $n = 4$).

C=O), 1649, 1602 (s, C=N). ^{1}H NMR: 8.21 (bs, 2H; NH_2), 7.97 (dd, $J = 6.8, 1.6$, 1H; H-7), 7.92–7.29 (m, 10H; Ar-H), 6.63 (d, $J = 6.5$, 1H; H-10). ^{13}C NMR: 193.2 (C-6), 175.3 (benzamide C=O), 170.3 (C-2), 165.7 (C-4), 142.9 (C-10a), 136.7 (C-4′), 136.2 (C-9), 134.7 (C-1′), 134 (C-1″), 133.5 (C-6a), 131.8 (C-8), 129.9 (C-2′), 128.7 (C-3′), 127.6 (C-3″), 125.5 (C-7), 125.4 (C-10), 125.1 (C-2″), 124.6 (C-4″), 116.3 (CN), 104.9 (C-11), 70.9 (C-5a), 54.1 (C-5). FAB MS: m/z (%) = 448 [M^+] (22). Anal. Calcd. for $C_{26}H_{16}N_4O_2S$ (448.10): C, 69.63; H, 3.60; N, 12.49; S, 7.15. Found: C, 69.79; H, 3.49; N, 12.52; S, 7.28.

(Z)-N-(E)-4-Amino-5-cyano-1-(4-methylphenyl)-6-oxo-1H-indeno[1,2-d][1,3]thiazepin-2(6H)-ylidene)-4-methylbenzamide (3b). Yellowish white crystals (DMF/H_2O, 10:1), yield = 381 mg (80%), m.p. 271–272°C. IR (KBr): 3295, 3120 (w, m, NH_2), 3070–3000 (m, Ar-CH), 2990–2910 (m, aliph.-CH), 2210 (s, CN), 1733, 1669 (s, s, C=O), 1645, 1608 (s, s, C=N). ^{1}H NMR: 8.22 (bs, 2H; NH_2), 7.98 (dd, $J = 6.6, 1.4$, 1H; H-7), 7.75 (t, $J = 5.7$, 2H; H-8,9), 7.7 (d, $J = 8.1$, 2H; H-2′), 7.43 (d, $J = 8.2$, 2H; H-3″), 7.33 (bd, $J = 7.2$, 2H; H-2″), 7.19 (d, $J = 8.0$, 2H; H-3′), 6.61 (d, $J = 6.8$, 1H; H-10), 2.47 (s, 3H; H-4a″), 2.3 (s, 3H; H-4a′). ^{13}C NMR: 193.4 (C-6), 175.2 (benzamide C=O), 170.1 (C-2), 165.9 (C-4), 143.6 (C-10a), 143.2 (C-4′), 139.1(C-4″), 136.5 (C-9), 133.7 (C-1″), 133.3 (C-6a), 132.6 (C-1′), 132.3 (C-8), 129.9 (C-3″), 129.2 (C-2′), 129.1 (C-2″), 129.0 (C-3′), 125.4 (C-7), 125.0 (C-10), 116.0 (CN), 105.3 (C-11), 70.6 (C-5a), 53.2 (C-5), 21.1 (C-4a′), 20.9 (C-4a″). MS (70 eV): m/z (%) = 476 [M^+] (24), 449 (23), 342 (14), 284 (12), 183 (10), 149 (20), 119 (100), 91 (40), 65 (18). Anal. Calcd. for $C_{28}H_{20}N_4O_2S$ (476.55): C, 70.57; H, 4.23; N, 11.76; S, 6.73. Found: C, 70.69; H, 4.29; N, 11.52; S, 6.58.

(Z)-N-(E)-4-Amino-5-cyano-6-oxo-1-phenyl-1H-indeno[1,2-d][1,3]thiazepin-2(6H)-ylidene)-4-methoxybenzamide (3c). Yellowish white crystals (acetone), yield = 407 mg, (85%), m.p. 270–271°C. IR (KBr): 3350, 3150 (w, NH_2), 3050–3013 (m, Ar-CH), 2996–2923 (m, aliph.-CH), 2200 (s, CN), 1727, 1665 (s, C=O), 1608 (s, C=N). ^{1}H NMR: 8.22 (bs, 2H; NH_2), 7.97 (d, $J = 7.4$, 1H; H-7), 7.75 (d, $J = 8.4$, 2H; H-2′), 7.71–7.68 (m, 2H; H-8,9), 7.63 (bs, 3H; H-3″,4″), 7.45 (bs, 2H; H-2″), 6.91 (d, $J = 8.7$, 2H; H-3′), 6.57 (d, $J = 7.5$, 1H; H-10), 3.77 (s, 3H; OCH_3). ^{13}C NMR: 193.4 (C-6), 174.6 (benzamide C=O), 169.6 (C-2), 165.9 (C-4), 163 (C-4′), 143.5 (C-10a), 136.5 (C-9), 136.4 (C-1″), 133.5 (C-6a), 132.6 (C-2′), 131.4 (C-8), 129.6 (C-2″), 129.5 (C-4″), 129.4 (C-3″), 127.4 (C-1′), 125.4 (C-7), 125.0 (C-10), 116.0 (CN), 113.7 (C-3′), 105.2 (C-11), 70.8 (C-5a), 55.4 (OCH_3), 53.2 (C-5). MS (70 eV): m/z (%) = 478 [M^+] (24), 451 (20), 387 (13), 285 (20), 119 (100), 91 (32), 65 (14). Anal. Calcd. for $C_{27}H_{18}N_4O_3S$ (478.52): C, 67.77; H, 3.79; N, 11.71; S, 6.70. Found: C, 67.97; H, 3.70; N, 11.79; S, 6.85.

(Z)-N-(E)-4-Amino-5-cyano-1-(4-metoxylphenyl)-6-oxo-1H-indeno[1,2-d][1,3]thiazepin-2(6H)-ylidene)-4-methylbenzamide (3d). Yellowish white crystals (DMF/H_2O, 10:1), yield = 404 mg, (82%), m.p. 278–280°C. IR (KBr): 3296, 3132 (m, NH_2), 3048–3011 (m, Ar-CH), 2992–2914 (m, aliph.-CH), 2212 (s, CN), 1731, 1670 (s, C=O), 1645, 1605 (s, C=N). ^{1}H NMR: 8.21 (bs, 2H; NH_2), 7.98 (d, $J = 8.5$, 1H; H-7), 7.78 (t, $J = 6.7$, 2H; H-8,9), 7.72 (d, $J = 8.0$, 2H; H-2′), 7.35 (bs, 2H; H-2″), 7.21 (d, $J = 8.0$, 2H; H-3′), 7.16 (d, $J = 8.2$, 2H; H-3″), 6.65 (d, $J = 5.1$, 1H; H-10), 3.89 (s, 3H; OCH_3), 2.31 (s, 3H; C—CH_3). ^{13}C NMR: δ_C 193.4 (C-6), 175.2 (benzamide C=O), 170.3 (C-2), 165.9 (C-4), 159.6 (C-4″), 143.6 (C-4′), 143.2 (C-10a), 136.5 (C-9), 133.4 (C-6a), 132.5 (C-8), 132.3 (C-1″), 130.7 (C-2″), 129.3 (C-2′), 129.0 (C-3′), 128.7 (C-1′), 125.4 (C-7), 125.1 (C-10), 116.0 (C-3″), 114.5 (CN), 105.4 (C-11), 70.5 (C-5a), 55.5 (OCH_3), 53.2 (C-5), 21.1 (C—CH_3). MS (70 eV): m/z (%) = 492 [M^+] (25), 465 (10), 342 (12), 300 (18), 266 (14), 208 (12), 183 (20), 165 (36), 119 (100), 91 (34), 65 (12). Anal. Calcd. for $C_{28}H_{20}N_4O_3S$ (492.55): C, 68.28; H, 4.09; N, 11.37; S, 6.51. Found: C, 68.00; H, 4.12; N, 11.20; S, 6.45.

(Z)-N-(E)-4-amino-5-cyano-1-(4-iodophenyl)-6-oxo-1H-indeno[1,2-d][1,3]thiazepin-2(6H)-ylidene)-4-methylbenzamide (3e). Yellowish white crystals (ethyl acetate), yield = 412 mg (70%), m.p. 294–295°C. IR (KBr): 3275, 3150 (m, NH_2), 3063–3013 (m, Ar-CH), 2988–2917 (m, aliph.-CH), 2202 (s, CN), 1732, 1669 (s, C=O), 1643, 1605 (s, C=N). ^{1}H NMR: 8.22 (bs, 2H; NH_2), 7.99 (d, $J = 8.2$, 2H; H-2″), 7.98–7.94 (m, 1H; H-7), 7.76–7.72 (m, 2H; H-8,9), 7.69 (d, $J = 7.7$, 2H; H-2′), 7.28 (d, $J = 7.6$, 2H; H-3″), 7.21 (d, $J = 7.7$, 2H; H-3′), 6.69–6.65 (m, 1H; H-10), 2.31 (s, 3H; C—CH_3). ^{13}C NMR: 193.2 (C-6), 175.2 (benzamide C=O), 170.2 (C-2), 165.9 (C-4), 143.4 (C-10a), 143.3 (C-4′), 138.3 (C-2″), 136.6 (C-9), 136.1 (C-1″), 133.4 (C-6a), 132.6 (C-1′), 132.2 (C-8), 131.5 (C-3″), 129.3 (C-2′), 129.0 (C-3′), 125.5 (C-7), 125.0 (C-10), 115.9 (CN), 105.0 (C-11), 96.2 (C-4″), 70.9 (C-5a), 53.1 (C-5), 21.2 (C—CH_3). MS (70 eV): m/z (%) = 588 [M^+] (12), 119 (100), 91 (12), 65 (11). Anal. Calcd. for $C_{27}H_{17}IN_4O_2S$ (588.42): C, 55.11; H, 2.91; N, 9.52; S, 5.45. Found: C, 55.24; H, 2.88; N, 9.28; S, 5.30.

Biological section

Cell culture. Human hepatocellular carcinoma (HepG2) cells were routinely cultured in Dulbeco's Modified Eagle's Medium. Media were supplemented with 10% fetal bovine serum, 2 mM L-glutamine, containing 100 units/mL penicillin G sodium, 100 units/mL streptomycin sulphate, and 250 ng/mL amphotericin B. Cells were maintained at subconfluency at 37°C in humidified air containing 5% CO_2. For subculturing, monolayer cells were harvested after trypsin/EDTA treatment at 37°C. Cells were used when confluence had reached 75%. Tested samples were dissolved in dimethyl sulphoxide (DMSO). All cell culture material was obtained from Cambrex BioScience (Copenhagen, Denmark). All chemicals were obtained from Sigma/Aldrich, USA, except mentioned. All experiments were repeated three times, unless mentioned.

Cytotoxicity assay. Cytotoxicity of tested samples was measured using the MTT cell viability assay. MTT (3-[4,5-

dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide) assay is based on the ability of active mitochondrial dehydrogenase enzyme of living cells to cleave the tetrazolium rings of the yellow MTT and form a dark blue insoluble formazan crystals, which is largely impermeable to cell membranes, resulting in its accumulation within healthy cells. Solubilization of the cells results in the liberation of crystals, which are then solubilized. The number of viable cells is directly proportional to the level of soluble formazan dark blue color. The extent of the reduction of MTT was quantified by measuring the absorbance at 570 nm [9].

Reagents preparation. MTT solution: 5 mg/mL of MTT dissolved in 0.9% NaCl. Acidified isopropanol: 0.04 N HCl in absolute isopropanol.

Procedure. Cells (0.5×10^5 cells/well) in serum-free media were plated in a flat bottom 96-well microplate, and treated with 20 μL of different concentrations of each tested compound for 20 h at 37°C in a humidified 5% CO_2 atmosphere. After incubation, media were removed and 40 μL MTT solution/well were added and incubated for an additional 4 h. MTT crystals were solubilized by adding 180 μL of acidified isopropanol/well, and the plate was shaken at room temperature, followed by photometric determination of the absorbance at 570 nm using a microplate ELISA reader. Triplicate repeats were performed for each concentration and the average was calculated. Data were expressed as the percentage of relative viability compared with the untreated cells compared with the vehicle control, with cytotoxicity indicated by <100% relative viability.

Calculations. Percentage of relative viability was calculated using the following equation:

$$[\text{Absorbance of treated cells}/\text{Absorbance of control cells} \times 100$$

Then the half maximal inhibitory concentration IC_{50} was calculated from the equation of the dose-response curve.

Antioxidant activity (scavenging of DPPH). DPPH is a stable deep violet radical because of its unpaired electron. In the presence of an antioxidant radical scavenger, which can donate an electron to DPPH, the deep violet color decolorizes to the pale yellow nonradical form [10]. The change of color and the subsequent fall in absorbance are monitored spectrophotometrically at $\nu = 520$ nm.

Reagents preparation. Ethanolic DPPH: 0.1 m*M* DPPH/absolute ethanol. Standard ascorbic acid solution: Serial dilutions of ascorbic acid in concentrations ranging from 0 to 2.5 μ*M* in distilled water. A standard calibration curve was plotted using serial dilutions of ascorbic acid in concentrations ranging from 0 to 2.5 μ*M* in distilled water.

Procedure. In a flat bottom 96 well-microplate, a total test volume of 200 μL was used. In each well, 20 μL of different concentrations (0–100 μg/mL final concentration) of tested compounds were mixed with 80 μL of ethanolic DPPH were mixed and incubated for 30 min at 37°C. Triplicate wells were prepared for each concentration and the average was calculated. Then photometric determination of absorbance at 515 nm was done using a microplate ELISA reader.

Calculations. The half-maximal scavenging capacity (SC_{50}) values for each tested compounds and ascorbic acid was estimated *via* two competitive dose curves.

$$\text{Abs}_{50} \text{ of ascorbic acid} = (\text{Abs}_{100} - \text{Abs}_0)/2$$

SC_{50} of ascorbic acid was calculated using the curve equation. SC_{50} of each compound was determined using the curve equation using Abs_{50} of ascorbic acid.

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