Full Paper

Thieno[2,3-*d*]pyrimidines in the Synthesis of Antitumor and Antioxidant Agents

Ashraf A. Aly[1], Alan B. Brown[2], Mohamed Ramadan[3], Amira M. Gamal-Eldeen[4], Mohamed Abdel-Aziz[3], Gamal El-Din A. A. Abuo-Rahma[3], and Mohamed F. Radwan[3]

[1] Chemistry Department, Faculty of Science, El-Minia University, El-Minia, Egypt
[2] Chemistry Department, Florida Institute of Technology, Melbourne, FL, USA
[3] Department of Medicinal Chemistry, Faculty of Pharmacy, El-Minia University, El-Minia, Egypt
[4] Department of Biochemistry, Division of Genetic Engineering and Biotechnology, National Research Centre, Dokki, Giza, Egypt

Dimethyl acetylenedicarboxylate, ethyl propiolate, and *E*-dibenzoylethylene react with thienopyrimidines (cyclo-pentyl, -hexyl, and -heptyl) derivatives to form thiazolo[3,2-*a*]thieno-[2,3-*d*]pyrimidin-2-ylidene) acetates, thieno[2,3-*d*]pyrimidin-2-ylthioacrylates, and thieno[2′,3′:4,5]pyrimido[2,1-*b*][1,3]thiazin-6-ones, respectively. Reactions proceed *via* cyclization and thio-addition processes. Some derivatives of thienopyrimidines showed high inhibition of Hep-G2 cell growth compared with the growth of untreated control cells. However, the fused heptyl of thienopyrimidothiazines indicates a promising specific antitumor agent against Hep-G2 cells with $IC_{50} < 20$ μM.

Keywords: Antitumor activity / Cyclization / Dimethyl acetylenedicarboxylate / *E*-Dibenzoylethylene / Thienopyrimidines

Received: October 11, 2009; Accepted: December 11, 2009

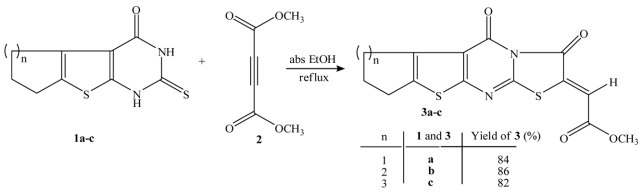
DOI 10.1002/ardp.200900245

Introduction

Thienopyrimidines are known to be of particular interest for the composition of some non-steroidal anti-inflammatory drugs (NSAIDs) [1]. Moreover, condensed heterocycles containing thienopyrimidines have acquired conspicuous popularity in recent years due to their wide spectrum of biological activities including analgesic [2–6], anti-inflammatory [3–8], antipyretic [4], antihypertensive [9, 10], pesticidal [11], herbicidal [12, 13], plant growth regulatory [13], spasmolytic [14], gastric antisecretory [15], antihistaminic [16], antibacterial [17–20], antifungal [21, 22], antimalarial [23], anti-HIV-1 and anti-Herpes simplex virus HSV-1 [24], antitumor [25, 26], as selective 5-HT_3 receptor ligands [27], hypnotics [28], and many more. Indeed, several series of heterocyclic compounds possessing a bridgehead thiazole or thiazine moiety play a vital role in many biological activities: thiazole derivatives such as pyrimidobenzothiazole and benzothiazoloquinoline derivatives, imidazobenzothiazoles as well as polymerized benzothiazoles showed remarkable antitumor activity [29]. On the other hand, 1,3-thiazines are a class of compounds of biological interest especially as antitumor and antioxidant agents [30]. The incorporation of two moieties may give a synergistic effect, so it was of value to synthesize novel heterocycles having two moieties in the same molecules [31]. In view of the aforementioned studies, the present work involves the synthesis of some novel heterocycles containing the thiazolo and thiazinothienopyrimidine systems, in the hope that they may exhibit a synergistic anticancer and/or antioxidant activity. In this paper, we investigate the reactions of thieno[2,3-*d*]pyrimidines **1a–c** with dimethyl acetylenedicarboxyate **2**, ethyl propiolate **6**, and (*E*)-dibenzoylethylene **9**. The antitumor and antioxidant activities of the obtained products were investigated.

Correspondence: Prof. Ashraf A. Aly, Chemistry Department, Faculty of Science, El-Minia University, 61519-El-Minia, Egypt.
E-mail: ashrafaly63@yahoo.com
Fax: +20 86 234-6876

Abbreviations: 1,1-diphenyl-2-picryl hydrazide (DPPH); (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide) (MTT); preparative thin layer chromatography (PLC)



Scheme 1. Synthesis of methyl(thieno[2′,3′:4,5]pyrimido[2,1-*b*][1,3]thiazol-2-ylidene **3a–c**.

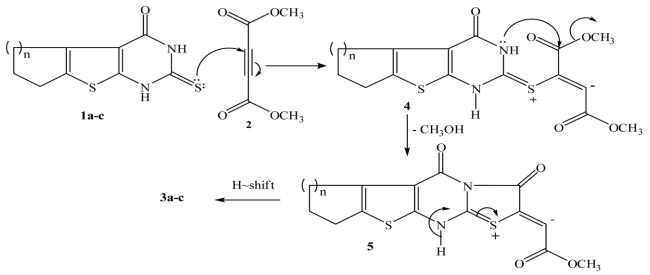
Results and Discussion

Chemistry

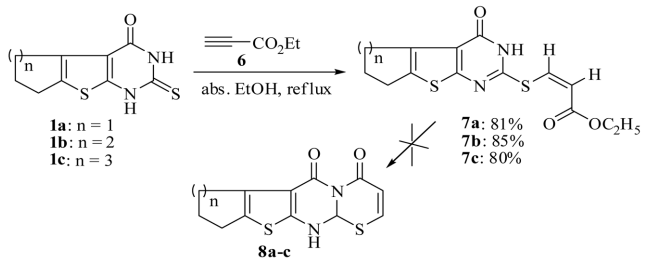
*Reaction of thienopyrimidines **1a–c** with dimethyl acetylenedicarboxylate **2***

Cycloalka[4,5][1,3]thiazolo[3,2-*a*]thieno[2,3-*d*]pyrimidines **3a–c** were synthesized through the reaction of thieno[2,3-*d*]pyrimidines **1a–c** with compound **2** in absolute ethanol (Scheme 1). The initial addition of the sulfur atom of thieno[2,3-*d*]pyrimidines **1a–c** to the acetylenic triple bond of compound **2**, would generate adducts **4a–c** which can release a molecule of methanol under nucleophilic attack by the amino group, to yield intermediates **5a–c** (Scheme 2). Proton shift is then proposed in **5a–c**, to produce the stable heterocycles **3a–c** as shown in Scheme 2. Based on previous reports, the *N*-3 and not the *N*-1 nitrogen atom of the thieno[2,3-*d*]pyrimidines was involved in the cyclization process to form the corresponding adduct [4, 32].

The mass spectra indicated a product from one molecule of **1a–c** and one molecule of **2** with the loss of MeOH. In **3c**, the magnitude of the coupling between C-3 and vinylic-H (J = 5.8 Hz) requires this to be a three-bond not two-bond coupling. Consequently, the C-3 and vinylic-H be mutually *cis*. The spectra contain one methoxy group signal at δ_H = 3.90 and δ_C = 53.0 ppm. This proton signal shows HMBC correlation with the signal at δ_C = 166.1 ppm, which shows three-bond quartet coupling with the methoxy protons, and is assigned as the ester C=O. The methoxy protons also show HMBC correlation with the vinylic carbon at δ_C = 120.2 ppm, which is assigned as C-2′ and its attached proton at δ_H = 7.19 ppm as H-2′. Two carbon signals show HMBC correlation and doublet coupling with H-2′: the vinylic carbon at δ_C = 139.6 ppm, assigned as C-2, and the carbonyl at δ_C = 161.7 ppm, assigned as C-3. The remaining carbonyl at δ_C = 158.9 ppm is assigned as C-5. In the cycloheptane ring, the combination of COSY correlations and chemical-shift simulation using CHEMNMR leads to the conclusion that the five methylenes are connected in the order δ_H = 3.29, 1.67, 1.89, 1.71, and 2.84 ppm. The correlation between δ_H = 3.29 and 2.84 ppm is assigned as a long-range coupling across the double bond.



Scheme 2. Plausible mechanism of heterocyclic formation of **3a–c**.

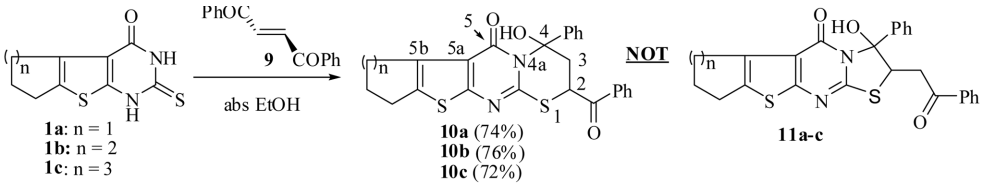


Scheme 3. Synthesis of (*Z*)-ethyl 3′-((4-oxo-cycloalka[5,6]-thieno[2,3-*d*]pyrimidin-2-yl)thio)acrylates **7a–c**.

The two vinylic carbons with extensive coupling into the cycloheptane ring (δ_C = 139.3 and 138.9 ppm) are assigned as C-10a and C-5b, and the two remaining downfield singlet carbons as C-12a and C-11a at (δ_C = 154.8 and 152.5 pm, respectively). The spectra of **3a**, **b** are very similar to those of **3c** with the same magnitude of coupling between C-3 and vinylic-H (J = 5.8 Hz).

*Reaction of thienopyrimidines **1a–c** with ethyl propiolate **6***

When compounds **1a–c** react with ethyl propiolate **6** in refluxing ethanol, (*Z*)-ethyl 3′-((4-oxo-cycloalka[4,5]-thieno[2,3-*d*]pyrimidin-2-yl)thio)acrylates **7a–c** were obtained (Scheme 3), via conjugate addition of the sulfur in **1a–c** to the triple bond of **6**. Surprisingly, the reaction stopped at this step to form compounds **7a–c**, without a further nucleophilic attack of the *N*-3 atom of **1a–c** on the carbonyl of **6** leading, as expected, to heterocycles **8a–c** (Scheme 3). The structure of the obtained products **7a–c** was confirmed by spectroscopic data and elemental analyses. The IR spectra of **7a–c** showed absorption bands at ν = 3417–3345 cm^{-1} characteristic of NH. The mass spectra of **7a–c** showed the molecular ion peaks, and elemental analysis confirmed the assigned molecular formulae of **7a–c**. The ^{1}H-NMR spectra of these compounds revealed broad signals at δ_H = 11.29–10.22 ppm characteristic of NH groups, which indicate that no cyclization occurred. The coupling constants between the vinylic protons of **7a–c** are 10 Hz, which require that the olefinic configurations be *Z*.



Scheme 4. Synthesis of thiazino[3,2-*a*]thieno[2,3-*d*]pyrimidine derivatives **10a–c** from the reaction of thieno[2,3-*d*]pyrimidines **1a–c** and (*E*)-1,4-diphenylbut-2-ene-1,4-dione **9**.

*Reaction of thienopyrimidines **1a–c** with (E)-dibenzoylethylene **9***

The reaction of thieno[2,3-*d*]pyrimidines **1a–c** with (*E*)-dibenzoylethylene **9** in refluxing ethanol produced 2-benzoyl-4-hydroxy-4-phenylcycloalka[4′,5′]thieno[2′,3′:4,5]pyrimido[2,1-*b*][1,3]thiazin-6–one derivatives **10a–c** in 72–76% yields (Scheme 4). The IR spectra showed the presence of OH at ν = 3520–3375 cm^{-1}. The NMR spectra of **10a–c** revealed that the products appeared to be a mixture of two isomers in a ratio of 3:1. The NH proton of compounds **7a–c** at δ_H = 11.29–10.22 ppm is absent, whilst the appearance of only one benzoyl carbonyl carbon and appearance of a broad singlet signal corresponding to OH at ~6.5 ppm can be attributed to the formation of a hemi-aminal structure, the other benzoyl carbonyl group having disappeared *via* cyclization. If the events begin with a conjugate attack of compound **1** on compound **9**, closure gives compound **10a** as a mixture of stereoisomers.

It is clear from the close resemblance to compounds **3a–c** and **7a–c** that the tricyclic substructure derived from compound **1** remains intact in compounds **10a–c**. Assignments are shown on structure **10a**, the C-11a gives HMBC correlation to the aliphatic methine (H-2); this proton is absent in compounds **3** and **7**.

The ^{1}H-coupled ^{13}C-NMR spectrum of **10a** contains eight signals for aromatic carbons. Four signals are twice as tall as the others are; the tall signals must be those representing two carbons each (2′,3′,5′,6′,2″,3″,5″,6″). Of these, the two double-triplets (δ_C = 128.2 and 124.4 ppm) must be C-2′ and C-2″, because each has two three-bond C-H couplings. One of these (δ_C = 128.2 ppm) shows HMQC correlation with the farthest downfield ^{1}H signal (δ_H = 8.00 ppm). This proton signal shows HMBC correlation with the benzoyl ketone-type ^{13}C signal at δ_C = 197.1 ppm. Thus, the signal at δ_H = 8.00 ppm and its attached carbon at δ_C = 128.2 ppm are assigned as H-2′,6′ and C-2′,6′, respectively. In the ^{13}C spectrum, one of the small aromatic double-triplets (δ_C = 133.6 ppm) is attributed to C-4′, because it gives HMBC correlation to H-2′. The attached proton at δ_H = 7.59 ppm is assigned as H-4′. The two ^{13}C double-doublets (δ_C = 129.0 and 128.7 ppm) must be C-3″,5″ and C-3′,5′, because each has only one three-bond C-H coupling; they are assigned in the order stated, because the latter is attached to protons at δ_H = 7.48 ppm, which, in turn, give COSY correlation to H-2′,6′. The attached protons appear at δ_H = 7.37 (H-3″,5″) and 7.48 ppm (H-3′,5′), respectively. C-1′ and C-1″ appear at δ_C = 141.5 and 136.2 ppm; the upfield of the two gives HMBC correlation with δ_H = 7.48 ppm and is assigned as C-1′, because these carbons of the three-bond couplings are with the *meta* protons. C-1″ gives HMBC correlation with H-3″. The double-triplet at δ_C = 129.2 ppm is assigned as C-4″; the attached proton gives a multiplet at δ_H = 7.43 ppm. The structural assignment hinges on the substructure derived from dibenzoylethylene. The hydroxylic proton at δ_H = 6.48 ppm gives HMBC correlation with C-2 but not C-3. In the major compound, both H-3 protons give NOESY correlation with H-2′, but neither gives correlation with H-2″. In the minor compound, one H-3 protons gives NOESY correlation with both H-2′ and H-2″. Correlation with H-2′ is uninformative; correlation with H-2″ suggests that in the minor compound, the phenacyl side chain is *cis* to the distal phenyl group. Therefore, the major compound is proposed to be (2*R**,4*S**)-**10a** (major), and the minor compound as (2*R**,4*R**)-**10a** (minor). The structures of both stereoisomers are shown in Fig. 1.

The benzoyl carbonyls of **10a–c** appear as triplets (perhaps actually triplet-triplets) with *J* = 4.0–5.7 Hz, and give HMBC correlation to both H-3 and H-2′. The *J* value requires that the coupling to H-3 be over three bonds not two [33, 34], consistent with thiazino[3,2-*a*]thieno[2,3-*d*]pyrimidin-5-ones **10a–c**, but inconsistent with the regioisomers, thiazolo[3,2-*a*]thieno[2,3-*d*]pyrimidin-5-ones **11a–c**.

Biological investigation

Anticancer activity

The cytotoxicity of compounds **3a–c**, **7a–c**, and **10a–c** was studied using two cell lines of solid tumor (Hep-G2 and HCT-116 cells), which were treated with different doses of the tested compounds and submitted to MTT assay. The yellow tetrazolium salt is reduced by the mitochondrial enzyme succinate dehydrogenase, present in living cells,

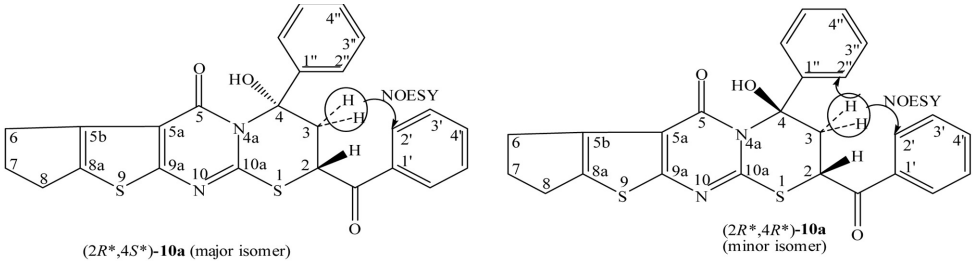


Figure 1. Structures of stereoisomer **10a**.

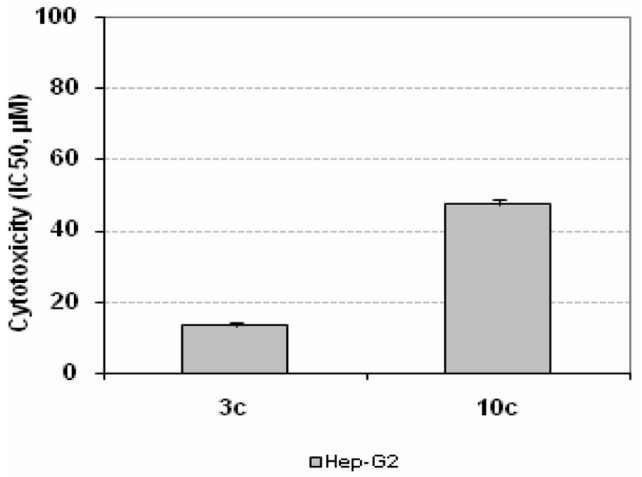


Figure 2. The effect of compounds **3c** and **10c** on the growth Hep-G2 cells, as measured by MTT assay.

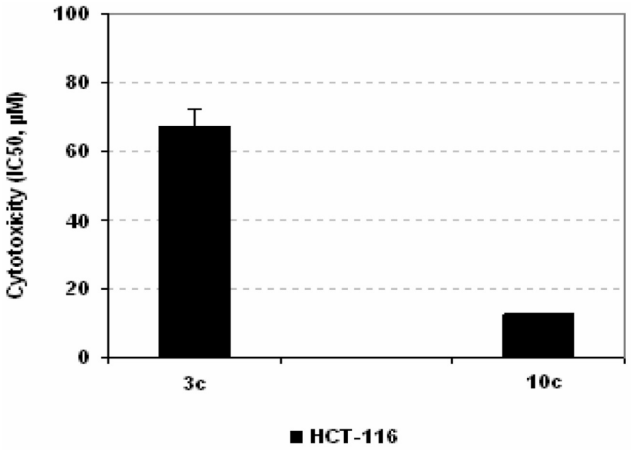


Figure 3. The cytotoxicity of compounds **3c** and **10c** against colon carcinoma cells (HCT-116), as measured by MTT assay. Results are represented as IC_{50} values (μM), mean ± S.E, $n = 4$.

to form insoluble formazan crystals, which are solubilized by the addition of detergent. The relative number of viable cells was determined by the amount of MTT converted to formazan crystals. The data were expressed as the mean percentage of the viable cells as compared to 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. Use of compounds **3c** and **10c** resulted in a significant inhibition of the cell growth of Hep-G2 cells compared with the growth of untreated control cells, as concluded from their low IC_{50} values 13.11 and 47.31 μM. Compound **3c** represents a promising specific antitumor agent against Hep-G2 cells as indicated from its low IC_{50} value <20 μM (Fig. 2).

Incubation of colon carcinoma HCT-116 cell line with gradual doses of the tested compounds resulted in an unchanged level of growth of HCT-116 cells, as indicated from their high IC_{50} values (>100 μM). However, compounds **3c** and **10c** which resulted in a high inhibition of the cell growth of HCT-116 cells compared with the growth of untreated control cells, as concluded from their IC_{50} values of 67.41 and 12.80 μM. Compound **10c** represents a promising specific antitumor agent against HCT-116 cells (IC_{50} is <20 μM) (Fig. 3).

Proliferation of T-lymphocytes and macrophages

Macrophages are the first line of defense against microbial infection; accordingly, the induction of macrophage proliferation is crucial in the assessment of the innate immunity. The effect of the compounds **3a–c**, **7a–c**, and **10a–c** on two types of immune cells, human lymphoblastic leukemia (1301, T-lymphocytes) and raw murine macrophage (RAW 264.7) was estimated by MTT assay using gradual doses of the tested compounds. Compounds **3c** and **10c** resulted in an insignificant inhibition in the 1301 cells: their IC_{50} values were >100 μM. Moreover, compounds **3a**, **7a**, **7c**, and **10a** exhibited no effect on the growth of 1301 cells. On the other hand, compounds **3b**, **7b**, and **10b** led to significant induction in the growth of 1301 cells up to 1.22- to 3.46-fold versus control, especially at high tested concentrations (50, 100 μM). Incubation of macrophages (RAW 264.7), for 48 h incubation with gradual doses of compound **3c** resulted in an insignificant inhibition in the macrophages: the IC_{50} value was >100 μM. Compounds **3a**, **7a**, **7c**, and **10b** exhibited

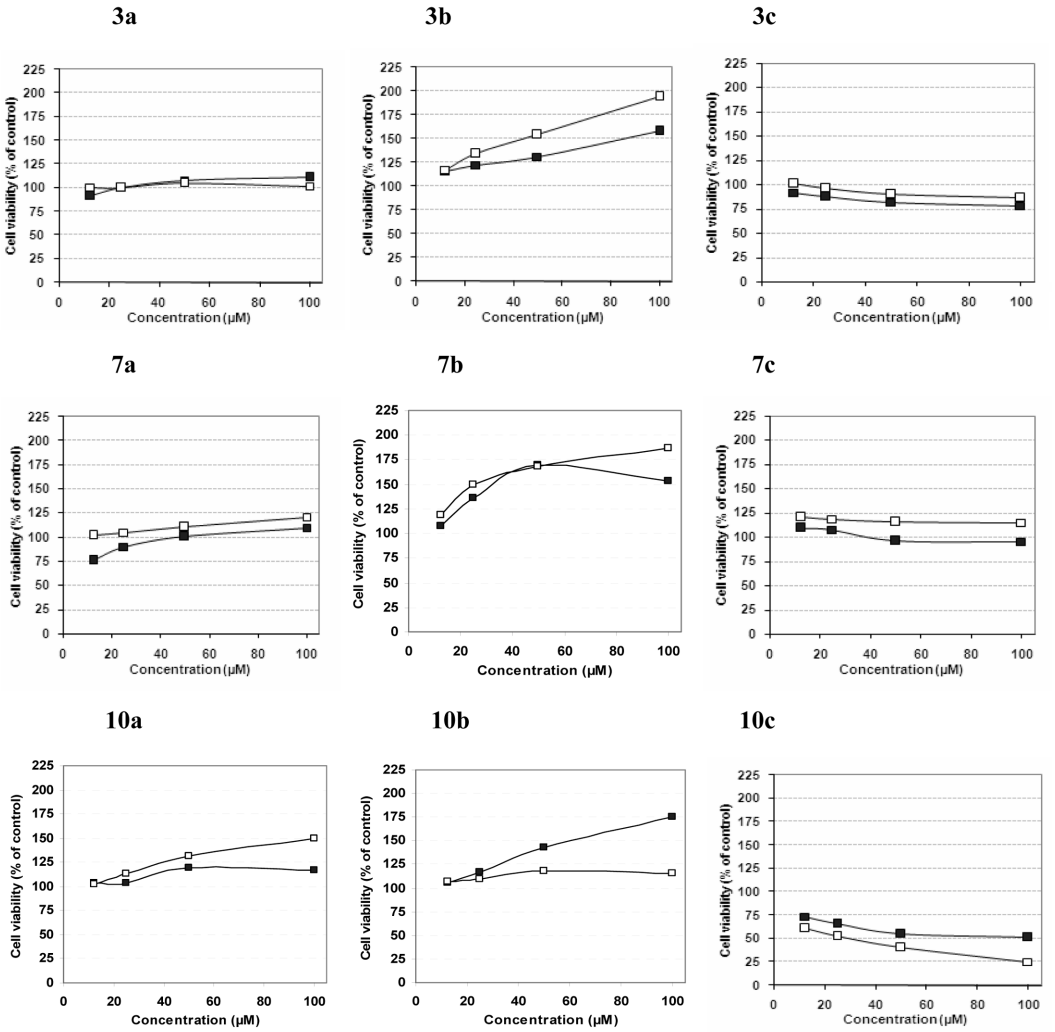


Figure 4. The effect of compounds **3a–c**, **7a–c**, and **10a–c** on the growth of two types of immune cells, human lymphoblastic leukemia (1301, T-lymphocytes, black squares-line) and Raw murine macrophage (RAW 264.7, white squares-line). As measured by MTT assay.

no effect on the growth of macrophages. On the other hand, compounds **3b**, **7b**, and **10a** led to significant induction in the growth of macrophages up to 1.18- to 3.99-fold versus control, especially at high tested concentrations (50, 100 μM) as shown in Fig. 4.

Antioxidant activity

The antioxidant capacity of compounds **3a–c**, **7a–c**, and **10a–c** was studied through their scavenging activity against 1,1-diphenyl-2-picryl hydrazide (DPPH). The bleaching of DPPH was monitored at absorbance ν = 515 nm. The percentage of DPPH bleaching was utilized for calculation of SC_{50} (half-maximal scavenging concentration).

Compounds **3b**, **3c**, **10a**, **10b**, and **10c** had effective antioxidant activity with SC_{50} values of 15.3, 86.4, 14.1, 13.2, and 26.4 μM respectively compared to the SC_{50} (8.41 μM) of the well-known antioxidant (ascorbic acid, A.A). On the other hand, compounds **3a** and **7a–c** possessed no scavenging activity to DPPH with high SC_{50} values (>100 μM) as shown in Fig. 5.

Conclusion

Compounds **3c** and **10c** show cytotoxicity against both types of solid tumor (Hep-G2 and HCT-116). However, **10c** and **3c** represent promising specific antitumor agents against HCT-116 cells and Hep-G2 cells, respectively (IC_{50} < 20 μM). Moreover, compounds **3b**, **7b**, and **10a** induced the growth of macrophages, while compounds **3b**, **7b**, and **10b** led to significant induction in the growth of

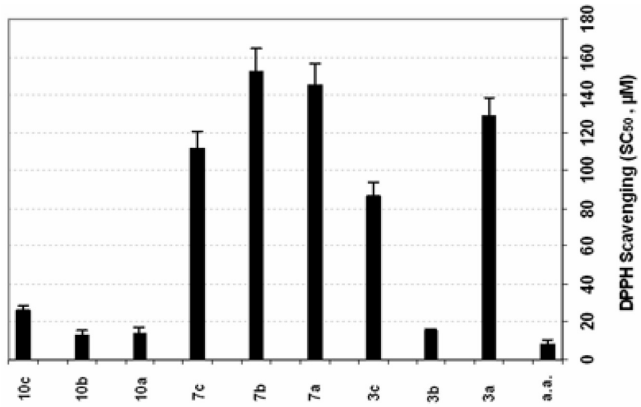


Figure 5. The antioxidant activity of the compounds **3a–c**, **7a–c**, and **10a–c** was investigated using DPPH assay. The results are represented as SC_{50} values (μM) as mean ± S.E, $n = 4$.

1301 cells. Compounds **3b** and **10a–c** were strong antioxidants. To elucidate the exact mechanism of these effects, the structure-activity relationship, pharmacological properties, and, to examine its therapeutic effects, further studies are required.

Experimental

Chemistry

Melting points are uncorrected. ^{1}H-NMR and ^{13}C-NMR spectra (Bruker AM 400 or AV-400; Bruker Bioscience, USA; ^{1}H: 400.13 MHz, ^{13}C: 100.6 MHz) were obtained from $CDCl_3$ and DMSO-d_6 solutions; chemical shifts (δ) are given relative to internal standard TMS, and coupling constants are stated in Hz. ^{1}H-coupled ^{13}C spectra were measured using gated decoupling; the notations CH, CH_2, and CH_3 refer to DEPT experiments. For preparative thin layer chromatography (PLC), glass plates (20 6 48 cm) were covered with a slurry of silica gel (Merck PF_{254}; Merck, Germany) and air-dried, using the solvents listed for development. Zones were detected by quenching of indicator fluorescence under 254 nm UV light. Elemental analyses were carried out using Vario EI Elementar, Microanalysis Center of National Research Center, Dokki, Giza, Egypt. Mass spectra were recorded on a Varian MAT 312 instrument (Varian, USA) in EI mode (70 eV.), Technische Universität Braunschweig, Germany; or by FAB on a JEOL JMS600 mass spectrometer (Jeol, Japan), Assiut University Central Lab, Assiut University, Assiut, Egypt. IR spectra were run on a Shimadzu 470 spectrometer (Shimadzu, Japan) using KBr pellets; absorption frequencies (ν) are stated in cm^{-1}.

Starting Materials: Dimethyl acetylenedicarboxylate **2** and ethyl propiolate **6** were purchased from Aldrich (Sigma-Aldrich); (*E*)-dibenzoylethylene **9** was purchased from Fluka (Sigma-Aldrich). Thieno[2,3-*d*]pyrimidines **1a–c** were prepared according to the literature [35].

*Reaction between thieno[2,3-d]pyrimidines **1a–c** and dimethyl acetylenedicarboxylate **2***

A mixture of **1a–c** (1 mmol) and **2** (142 mg, 1 mmol) was heated at reflux in absolute ethanol (30 mL) for 20–40 min; the reaction was followed by TLC. The orange precipitates were filtered, dried, and recrystallized from ethyl acetate.

*(Z)-Methyl (3,4-dioxo-6,7-dihydro-5H-cyclopenta[4,5][1,3]thiazolo[3,2-a]thieno[2,3-d]pyrimidin-2-ylidene)acetate **3a***

Orange crystals, yield: 281 mg (84%), m.p.: 232–234°C; IR (KBr) ν: 3060–3045 (vinylic-CH), 2970–2880 (aliph.-CH), 1762 (ester C=O), 1702, 1685 (pyrimidine C=O), 1557 (C=C); ^{1}H-NMR (400.13 MHz, $CDCl_3$): 7.20 (s, 1H, vinylic-H), 3.91 (s, 3H, OCH_3), 3.06 (t, J = 7.2, 2H, H-7), 2.96 (t, J = 7.3, 2H, H-5), 2.48 (quin, J = 7.3, 2H, H-6); ^{13}C-NMR (100.6 MHz, $CDCl_3$): 166.1 (q, J = 4.0, ester C=O), 165.3 (s, C-4), 161.6 (d, J = 5.8, C-3), 154.1 (s, C-9a), 152.5 (s, C-8a), 141.9 (m, C-7a), 140.2 (m, C-4b), 139.4 (d, J = 1.0, C-2), 120.4 (d, J = 174.4, C-2′), 117.8 (s, C-4a), 53.0 (q, J = 148.1, OCH_3), 29.6 (tt, J = 134.4, 2, C-5), 28.9 (tt, J = 134.1, 2, C-7), 27.9 (quin, J = 3.0, C-6); EI MS m/z (%): 334 [M^+] (100), 246 (30), 190 (26), 134 (7), 85 (11). Anal. calcd. for $C_{14}H_{10}N_2O_4S_2$ (334.37): C, 50.29; H, 3.01; N, 8.38; S, 19.18. Found: C, 50.08; H, 3.27; N, 8.42; S, 19.09.

*(Z)-Methyl (3,4-dioxo-5,6,7,8-tetrahydrobenzo[4,5][1,3]thiazolo[3,2-a]thieno[2,3-d]pyrimidin-2-ylidene)acetate **3b***

Orange crystals, yield: 300 mg (86%), m.p.: 255–257°C; IR (KBr) ν: 3060–3042 (vinylic-CH), 2970–2880 (aliph.-CH), 1775 (ester C=O), 1712, 1695 (pyrimidine C=O); ^{1}H-NMR (400.13 MHz, $CDCl_3$): 7.20 (s, 1H, vinylic-H), 3.91 (s, 3H, OCH_3), 2.98–2.95 (m, 2H, H-8), 2.76–2.71 (m, 2H, H-5), 1.87–1.82 (m, 4H, H-6,7); ^{13}C-NMR (100.6 MHz, $CDCl_3$): 166.1 (ester C=O), 161.7 (C-3), 160.6 (C-4), 154.2 (C-10a), 153.0 (C-9a), 139.5 (C-2), 135.0 (C-8a), 133.3 (C-4b), 120.3 (C-2′), 120.1 (C-4a), 53.0 (OCH_3), 25.4 (C-8), 25.1 (C-5), 22.75 (C-7), 22.0 (C-6); FAB MS m/z (%): 349 [M + 1] (20). Anal. calcd. for $C_{15}H_{12}N_2O_4S_2$ (348.4): C, 51.71; H, 3.47; N, 8.04; S, 18.41. Found: C, 51.61; H, 3.62; N, 8.05; S, 18.14.

*(Z)-Methyl (3,4-dioxo-6,7,8,9-tetrahydro-5H-cyclohepta[4,5][1,3]thiazolo[3,2-a]thieno-[2,3-d]pyrimidin-2-ylidene)acetate **3c***

Orange crystals, yield: 297 mg (82%), m.p.: 224–226°C; IR (KBr) ν: 2979–2895 (aliph.-CH), 1772 (ester C=O), 1710, 1690 (pyrimidine C=O), 1560 (C=C); ^{1}H-NMR (400.13 MHz, $CDCl_3$): 7.19 (s, 1H, vinylic-H), 3.90 (s, 3H, OCH_3), 3.29–3.27 (m, 2H, H-5), 2.84–2.82 (m, 2H, H-9), 1.89–1.87 (m, 2H, H-7), 1.71–1.64 (m, 4H, H-6,8); ^{13}C-NMR (100.6 MHz, $CDCl_3$): 166.1 (q, J = 3.7, ester C=O), 161.7 (d, J = 5.8, C-3), 158.9 (s, C-4), 154.8 (s, C-11a), 152.5 (s, C-10a), 139.6 (d, J = 1.1, C-2), 139.3 (quin, J = 7.5, C-9a), 138.9 (quin, J = 6.3, C-4b), 120.6 (t, J = 3.1, C-4a), 120.2 (d, J = 174.3, C-2′), 53.0 (q, J = 148.1, OCH_3), 32.4 (t of m, J_t = 121.6, C-7), 29.9 (t of m, J_t = 128.1, C-9), 27.7 (t of m, J_t = 130.2, C-5), 27.6 (t of m, C-8), 27.0 (t of m, J_t = 128.3, C-6); FAB MS m/z (%): 363 [M + 1] (100). Anal. calcd. for $C_{16}H_{14}N_2O_4S_2$ (362.42): C, 53.02; H, 3.89; N, 7.73; S, 17.69. Found: C, 52.62; H, 4.01; N, 7.68; S, 17.46.

*Reaction between thieno[2,3-d]pyrimidines **1a–c** and ethyl propiolate **6***

A mixture of **1a–c** (1 mmol) and **6** (98 mg, 1 mmol) was heated at reflux in absolute ethanol (30 mL) for 2–3 h; the reaction was followed by TLC analysis. The solvent was then removed under vacuum and the residue was separated by PLC (toluene/ethyl ace-

tate, 10:2). The major zones were extracted with acetone and the obtained products **7a–c** were recrystallized from ethyl acetate.

*(Z)-Ethyl 3′-((4-oxo-6,7-dihydro-3H,5H-cyclopenta[4,5] thieno[2,3-d]pyrimidin-2-yl)thio)acrylate **7a***

Yellowish white crystals, yield: 261 mg (81%), m. p.: 151–152°C; IR (KBr) ν: 3345 (NH), 3179–3168 (vinylic-CH), 2995–2880 (aliph.-CH), 1705 (ester C=O), 1675 (pyrimidine C=O), 1590 (C=N), 1545 (C=C); ^{1}H-NMR (400.13 MHz, DMSO-d_6): 10.22 (bs, 1H, NH), 8.32 (d, J = 10.0, 1H, H-3′), 6.22 (d, J = 10.2, 1H, H-2′), 4.28 (q, J = 7.2, 2H, CH_2CH$_3$), 3.10 (t, J = 7.2, 2H, H-7), 2.97 (t, J = 7.1, 2H, H-5), 2.47 (quin, J = 7.2, 2H, H-6), 1.34 (t, J = 7.2, 3H, CH$_2$CH_3); ^{13}C-NMR (100.6 MHz, DMSO-d_6): 167.9 (ester C=O), 166.6 (C-4), 158.1 (C-2), 150.7 (C-8a), 140.3 (C-3′), 138.4 (C-7a), 137.8 (C-4b), 118.1 (C-4a), 116.5 (C-2′), 61.1 (CH_2CH$_3$), 29.5 (C-5), 28.9 (C-7), 28.0 (C-6), 14.3 (CH$_2$CH_3); EI MS: m/z (%): 322 [M$^+$] (26), 250 (10), 248 (100), 191 (7), 105 (5). Anal. calcd. for $C_{14}H_{14}N_2O_3S_2$ (322.40): C, 52.16; H, 4.38; N, 8.69; S, 19.89. Found: C, 51.92; H, 4.28; N, 8.63; S, 20.0.

*(Z)-Ethyl 3′-((4-oxo-5,6,7,8-tetrahydro-3H-benzo[4,5]thieno[2,3-d]pyrimidin-2-yl)thio)acrylate **7b***

Yellowish white crystals, yield: 286 mg (85%), m. p.: 206–207°C; IR (KBr) ν: 3417 (NH), 3065 (vinylic-CH), 2960–2860 (aliph.-CH), 1700 (ester C=O), 1669 (pyrimidine C=O), 1595 (C=N), 1549 (C=C); ^{1}H-NMR (400.13 MHz, CDCl$_3$): 11.26 (bs, 1H, NH), 8.32 (d, J = 10.0, 1H, H-3′), 6.19 (d, J = 10.1, 1H, H-2′), 4.28 (q, J = 7.1, 2H, CH_2CH$_3$), 3.03–2.97 (m, 2H, H-8), 2.76–2.70 (m, 2H, H-5), 1.89–1.84 (m, 4H, H-6,7), 1.35 (t, J = 7.1, 3H, CH$_2$CH_3); ^{13}C-NMR (100.6 MHz, CDCl$_3$): 166.5 (dq, J_d = 12.9, J_q = 2.6, ester C=O), 163.2 (s, C-4), 159.3 (s, C-9a), 151.4 (d, J = 6.7, C-2), 138.1 (dd, J = 181.6, 5.4, C-3′), 133.1 (quin, J = 4.3, C-8a), 131.6 (quin, J = 3.7, C-4b), 120.5 (s, C-4a), 116.4 (d, J = 169.7, C-2′), 61.0 (tq, J_t = 147.8, J_q = 4.5, CH_2CH$_3$), 25.4 (bt, J = 130.2, C-8), 25.1 (bt, J = 129.1, C-5), 23.0 (t of quin, J_t = 129.0, J_{quin} = 3.9, C-7), 22.2 (t of quin, J_t = 128.9, J_{quin} = 3.5, C-6), 14.3 (tq, J_t = 2.4 Hz, J_q = 127.1, CH$_2$CH_3); FAB MS m/z (%): 337 [M + 1] (100). Anal. calcd. for $C_{15}H_{16}N_2O_3S_2$ (336.43): C, 53.55; H, 4.79; N, 8.33; S, 19.06. Found: C, 53.35; H, 4.93; N, 8.26; S, 18.88.

*(Z)-Ethyl 3′-((4-oxo-6,7,8,9-tetrahydro-3H,5H-cyclohepta[4,5]thieno[2,3-d]pyrimidin-2-yl)thio)-acrylate **7c***

Yellowish white crystals, yield: 280 mg (80%), m. p.: 224–226°C; IR (KBr) ν: 3409 (NH), 3050 (vinylic-CH), 2969–2872 (aliph.-CH), 1697 (ester C=O), 1660 (pyrimidine C=O), 1593 (C=N), 1545 (C=C); ^{1}H-NMR (400.13 MHz, CDCl$_3$): 11.29 (bs, 1H, NH), 8.37 (d, J = 10.2, 1H, H-3′), 6.20 (d, J = 10.2, 1H, H-2′), 4.23 (q, J = 7.2, 2H, CH_2CH$_3$), 3.28–3.24 (m, 2H, H-5), 2.82 (t, J = 5.4, 2H, H-9), 1.89–1.84 (m, 2H, H-7), 1.67–1.62 (m, 4H, H-6,8), 1.31 (t, J = 6.9, 3H, CH$_2$CH_3); ^{13}C-NMR (100.6 MHz, CDCl$_3$): 171.0 (ester C=O), 165.6 (C-4), 164.0 (C-2), 156.5 (C-10a), 143.7 (C-3′), 141.5 (C-9a), 140.8 (C-4b), 125.8 (C-4a), 120.5 (C-2′), 65.4 (CH_2CH$_3$), 37.2 (C-7), 34.4 (C-9), 32.5 (C-5), 32.4 (C-8), 32.0 (C-6), 19.1 (CH$_2$CH_3); FAB MS m/z (%): 351 [M + 1] (100). Anal. calcd. for $C_{16}H_{18}N_2O_3S_2$ (350.46): C, 54.83; H, 5.18; N, 7.99; S, 18.30. Found: C, 54.44; H, 4.97; N, 7.98; S, 18.16.

*Reaction between thieno[2,3-d]pyrimidines **1a–c** and (E)-dibenzoylethylene **9***

To a magnetically stirred solution of **1a–c** (1 mmol) in absolute ethanol (25 mL), compound **9** (236 mg, 1 mmol) in absolute ethanol (10 mL) was added. The mixture was heated under reflux for 6–9 h; TLC followed the reaction. The solvent was evaporated under reduced pressure and the residue was purified by PLC using toluene/ethyl acetate (10:1). The obtained products **10a–c** were recrystallized from ethyl acetate.

*2-Benzoyl-4-Hydroxy-4-phenyl-2,3,7,8-tetrahydro-6H-cyclopenta[4,5][1,3]thiazino[3,2-a]thieno-[2,3-d] pyrimidine-5(4H)one **10a***

Yellow crystals, yield: 340 mg (74%), m.p.: 239–241°C; IR (KBr) ν: 3500–3375 (OH), 3108–3046 (Ar-CH), 2979–2905 (aliph.-CH), 1679 (benzoyl C=O), 1658 (pyrimidine C=O), 1595 (C=N), 1543 (C=C); FAB MS m/z (%): 461 [M + 1] (73). Anal. calcd. for $C_{25}H_{20}N_2O_3S_2$ (460.57): C, 65.20; H, 4.38; N, 6.08; S, 13.92. Found: C, 65.48; H, 4.45; N, 5.68; S, 14.04.

Major isomer: ^{1}H-NMR (400.13 MHz, CDCl$_3$): 8.0 (d, J = 7.4, 2H, H-2′), 7.59 (t, J = 7.3, 1H, H-4′), 7.48 (t, J = 7.7, 2H, H-3′), 7.43–7.41 (m, 1H, H-4″), 7.37–7.33 (m, 4H, H-2″,3″), 6.48 (bs, 1H, OH), 4.36 (dd, J = 9.3, 4.9, 1H, H-2), 4.15 (dd, J = 18.4, 4.9, 1H, H-3), 3.54 (dd, J = 18.3, 9.5, 1H, H-3), 2.94–2.90 (m, 4H, H-6,8), 2.42 (quin, J = 7.0, 2H); ^{13}C-NMR (100.6 MHz, CDCl$_3$): 197.1 (t, J = 4.0, benzoyl C=O), 170.0 (s, C-5), 159.3 (s, C-9a), 157.2 (bs, C-10a), 141.5 (m, C-1″), 139.7 (m, C-8a), 138.0 (m, C-5b), 136.2 (t, J = 7.1, C-1′), 133.6 (dt, J_d = 162.1, J_t = 7.6, C-4′), 129.2 (dt, J_d = 160.8, J_t = 8.2, C-4″), 129.0 (dd, J = 161.6, 7.0, C-3″), 128.7 (dd, J = 161.9, 7.5, C-3′), 128.2 (dt, J_d = 160.2, J_t = 7.0, C-2′), 124.4 (ddd, J = 158.9, 6.5, 5.1, C-2″), 117.0 (s, C-5a), 98.1 (m, C-4), 51.3 (dm, J_d = 144, C-2), 41.0 (dt, J_d = 4.3, J_t = 127.5, C-3), 29.5 (tm, J_t = 135, C-8), 28.9 (tm, J_t = 133, C-6), 27.9 (tm, J_t = 132, C-7).

Minor isomer: ^{1}H-NMR (400.13 MHz, CDCl$_3$): 7.72 (d, J = 7.6, 2H, H-2′), 7.55 (t, J = 7.2, 1H, H-4′), 7.48 (t, J = 7.7, 2H, H-3′), 7.43–7.40 (m, 1H, H-4″), 7.37–7.32 (m, 4H, H-2″,3″), 6.55 (bs, 1H, OH), 4.76 (dd, J = 11.5, 2.9, 1H, H-2), 4.15 (dd, J = 17.5, 11.5, 1H, H-3), 3.51 (dd, J = 17.5, 3.4, 1H, H-3), 2.94–2.90 (m, 4H, H-6,8), 2.42 (quin, J = 7.0, 2H, H-7); ^{13}C-NMR (100.6 MHz, CDCl$_3$): 196.1 (q, benzoyl C=O), 170.0 (s, C-5), 158.8 (s, C-9a), 157.2 (bs, C-10a), 141.5 (m, C-1″), 139.7 (m, C-8a), 138.0 (m, C-5b), 135.5 (t, C-1′), 133.9 (CH, C-4′), 129.7 (CH, C-4″), 129.1 (CH, C-3″), 128.7 (CH, C-3′), 128.0 (CH, C-2′), 125.4 (CH, C-2″), 117.1 (s, C-5a), 99.2 (m, C-4), 49.9 (CH, C-2), 40.0 (CH$_2$, C-3), 29.5 (CH$_2$, C-8), 28.8 (CH$_2$, C-6), 27.9 (CH$_2$, C-7).

*2-Benzoyl-4-Hydroxy-4-phenyl-2,3,6,7,8,9-hexahydrobenzo[4,5][1,3]thiazino[3,2-a]thieno[2,3-d]-pyrimidine-5(4H)one **10b***

Yellow crystals, yield: 360 mg (76%), m.p.: 208–210°C; IR (KBr) ν: 3520–3398 (OH), 3098–3034 (Ar-H), 2985–2899 (aliph.-CH), 1670 (benzoyl C=O), 1655 (pyrimidine C=O), 1598 (C=N), 1535 (C=C); FAB MS m/z (%): 475 [M + 1] (34). Anal. calcd. for $C_{26}H_{22}N_2O_3S_2$ (474.59): C, 65.80; H, 4.67; N, 5.90; S, 13.51. Found: C, 65.49; H, 4.54; N, 5.68; S, 13.23.

Major isomer: ^{1}H-NMR (400.13 MHz, CDCl$_3$): 8.00 (d, J = 7.2, 2H, H-2′), 7.59 (t, J = 7.4, 1H, H-4′), 7.48 (t, J = 7.7, 2H, H-3′), 7.43 (t, J = 8.3, 1H, H-4″), 7.37–7.33 (m, 4H, H-2″,3″), 6.52 (bs, OH), 4.35 (dd, J = 9.3, 5.0, 1H, H-2), 4.15 (dd, J = 18.4, 5.0, 1H, H-3), 3.53 (dd, J = 18.4, 9.3, 1H, H-3), 2.87–2.84 (m, 2H, H-9), 2.75–2.72 (m, 2H, H-6), 1.86–1.81 (m, 2H, H-8), 1.79–1.75 (m, 2H, H-7); ^{13}C NMR (100.6 MHz, CDCl$_3$): 197.1 (t, J = 5.7, benzoyl C=O), 164.9 (s, C-5), 159.6 (s, C-10a), 157.6 (d, J = 4.4, C-11a), 141.5 (d, J = 2.9, C-1″), 136.2 (t, J = 6.9, C-1′), 133.6 (dt, J_d = 161.3, J_t = 8.1, C-4′), 132.7 (m, C-9a), 131.2 (m, C-5b), 129.2 (dt, J_d = 160, J_t = 6.7, C-4″), 129 (dd, J = 161.5, 7.2, C-3″), 128.7 (dd, J = 162.0, 7.5, C-3′), 128.2 (dt, J_d = 160.5, J_t = 6.9, C-2′), 124.5 (dt, J_d = 165.4, J_t = 5.9, C-2″), 119.4 (s, C-5a), 98.1 (m, C-4),

51.2 (dm, J_d = 149, C-2), 41.1 (dt, J_d = 3.1, J_t = 129.1, C-3), 25.4 (tm, J_t = 127.9, C-9), 25.1 (tm, J_t = 129.8, C-6), 22.9 (tm, J_t = 126.6, C-8), 22.1 (tm, J_t = 128.7, C-7).

Minor isomer: ^{1}H-NMR (400.13 MHz, $CDCl_3$): 7.72 (d, *J* = 7.3, 2H, H-2′), 7.55 (t, *J* = 7.4, 1H, H-4′), 7.48 (t, *J* = 7.7, 2H, H-3′), 7.43 (t, *J* = 8.3, 1H, H-4″), 7.37 (m, 4H, H-2″,3″), 6.59 (bs, OH), 4.75 (dd, *J* = 11.6, 2.9, 1H, H-2), 4.15 (dd, *J* = 18.4, 5.0, 1H, H-3), 3.70 (dd, *J* = 18.0, 4.2, 1H, H-3), 2.96–2.94 (m, 2H, H-9), 2.75–2.72 (m, 2H, H-6), 1.86–1.81 (m, 2H, H-8), 1.79–1.75 (m, 2H, H-7); ^{13}C-NMR (100.6 MHz, $CDCl_3$): 196.2 (benzoyl C=O), 164.9 (C-5), 158.9 (C-10a), 157.6 (C-11a), 141.5 (C-1″), 135.1 (C-1′), 133.7 (C-4′), 132.6 (C-9a), 131.1 (C-5b), 129.6 (C-4″), 129.2 (C-3″), 129.1 (C-3′), 128.0 (C-2′), 125.4 (C-2″), 119.6 (C-5a), 99.3 (C-4), 49.8 (C-2), 40.0 (C-3), 25.3 (C-9), 25.0 (C-6), 22.9 (C-8), 22.1 (C-7).

2-Benzoyl-4-Hydroxy-4-phenyl-2,3,7,8,9,10-hexahydro-6H-cyclohepta[4,5][1,3]thiazino[3,2-a]-thieno[2,3-d] pyrimidine-5(4H)one ***10c***

Yellow crystals, yield: 352 mg (72%), m. p.: 216–218°C; IR (KBr) ν: 3518–3395 (OH), 3097–3019 (Ar-CH), 2990–2874 (aliph.-CH), 1665 (benzoyl C=O), 1645 (pyrimidine C=O), 1599 (C=N), 1532 (C=C); FAB MS *m/z* (%): 489 [M + 1] (30). Anal. calcd. for $C_{27}H_{24}N_2O_3S_2$ (488.62): C, 66.37; H, 4.95; N, 5.73; S, 13.12. Found: C, 66.19; H, 4.85; N, 5.74; S, 13.38.

Major isomer: ^{1}H-NMR (400.13 MHz, $CDCl_3$): 8.00 (d, *J* = 7.4, 2H, H-2′), 7.59 (t, *J* = 7.5, 1H, H-4′), 7.48 (t, *J* = 7.8, 2H, H-3′), 7.44 (t, *J* = 8.5, 1H, H-4″), 7.37–7.32 (m, 4H, H-2″,3″), 6.53 (bs, OH), 4.33 (dd, *J* = 9.3, 5.0, 1H, H-2), 4.14 (dd, *J* = 18.3, 4.9, 1H, H-3), 3.52 (dd, *J* = 18.3, 9.4, 1H, H-3), 3.18–3.14 (m, 2H, H-6), 2.82–2.79 (m, 2H, H-10), 1.70–1.68 (m, 2H, H-8), 1.66–1.64 (m, 2H, H-9), 1.60–1.57 (m, 2H, H-7); ^{13}C-NMR (100.6 MHz, $CDCl_3$): 197.1 (benzoyl C=O), 163.3 (C-5), 160.0 (C-11a), 157.2 (C-12a), 141.6 (C-1″), 136.9 (C-1′), 136.8 (C-4′), 136.2 (C-10a), 133.6 (C-5b), 129.2 (C-4″), 129.0 (C-3″), 128.7 (C-3′), 128.2 (C-2′), 124.5 (C-2″), 120.0 (C-5a), 98.2 (C-4), 51.2 (C-2), 41.1 (C-3), 32.4 (C-8), 29.9 (C-10), 27.8 (C-6), 27.7(C-9), 27.1 (C-7).

Minor isomer: ^{1}H-NMR (400.13 MHz, $CDCl_3$): 7.71 (d, *J* = 7.5, 2H, H-2′), 7.55 (t, *J* = 7.4, 1H, H-4′), 7.48 (t, *J* = 7.8, 2H, H-3′), 7.44 (t, *J* = 8.5, 1H, H-4″), 7.37–7.32 (m, 4H, H-2″,3″), 6.61 (bs, OH), 4.74 (dd, *J* = 11.6, 2.8, 1H, H-2), 4.14 (dd, *J* = 18.3, 4.9, 1H, H-3), 3.71 (dd, *J* = 19.0, 6.2, 1H, H-3), 3.18–3.14 (m, 2H, H-6), 2.82–2.79 (m, 2H, H-10), 1.70–1.68 (m, 2H, H-8), 1.66–1.64 (m, 2H, H-9), 1.60–1.57 (m, 2H, H-7); ^{13}C-NMR (100.6 MHz, $CDCl_3$): 196.2 (benzoyl C=O), 163.3 (C-5), 160.0 (C-11a), 157.1 (C-12a), 141.7 (C-1″), 136.9 (C-1′), 136.7 (C-4′), 136.2 (C-10a), 133.9 (C-5b), 129.2 (C-4″), 129.1 (C-3″), 128.7 (C-3′), 128.0 (C-2′), 125.3 (C-2″), 120.2 (C-5a), 99.3 (C-4), 49.8 (C-2), 40.0 (C-3), 32.4 (C-8), 29.9 (C-10), 27.8 (C-6), 27.7 (C-9), 27.1 (C-7).

Biological section

Cell culture

Hepatocellular carcinoma (HepG2) and colon carcinoma HCT-116 were routinely cultured in DMEM (Dulbeco's Modified Eagle's Medium). Media were supplemented with 10% fetal bovine serum (FBS), 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 from Sigma/Aldrich, USA, except mentioned. All experiments were repeated three times, unless mentioned.

Cytotoxicty assay

Cytotoxicity of tested samples against Hepatocellular carcinoma (HepG2) colon carcinoma HCT-116 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 ν = 570 nm [36].

Reagents preparation

MTT solution: 5 mg/mL of MTT in 0.9% of 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 per 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 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 were calculated using the following equation:

$$[\text{Absorbance of treated cells}/\text{Absorbance of control cells}] \times 100 \quad (1)$$

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

Antioxidant activity (scavenging of DPPH)

1,1-Diphenyl-2-picrylhydrazyl is a stable deep violet radical due to 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 non-radical form [37]. The change in colorization and the subsequent fall in absorbance are monitored spectrophotometrically at ν = 520 nm.

Reagents preparation and standard ascorbic acid solution

Ethanolic DPPH: 0.1 mM DPPH/absolute ethanol.

Serial dilutions of ascorbic acid in concentrations ranging from 0 to 2.5 μM in distilled water. A standard calibration curve was plotted using serial dilutions of ascorbic acid in concentrations ranging from 0 to 2.5 μM 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 180 μL of ethanolic DPPH 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 made, 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. Abs_{50} of ascorbic acid = $(Abs_{100} - Abs_0)/2$.

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

Purchase of the AV-400 NMR spectrometer was assisted by the National Science Foundation (CHE 03-42251).

The authors have declared no conflict of interest.

References

[1] F. Russo, A. Santagati, M. Santagati, A. Caruso, *et al*., *Eur. J. Med. Chem.* **1989**, *24*, 91 – 95.

[2] M. Perrissin, M. Favre, C. Luu-Duc, F. Huguet, *et al*., *Eur. J. Med. Chem.* **1988**, *23*, 453 – 456.

[3] A. Cannito, M. Perrissin, C. Luu-Duc, F. Huguet, *et al*., *Eur. J. Med. Chem.* **1990**, *25*, 635 – 639.

[4] A. B. A. El-Gazzar, H. A. R. Hussein, H. N. Hafez, *Acta Pharm. (Zagreb)* **2007**, *57*, 395 – 411.

[5] S. Vega, J. Alonso, J. A. Diaz, F. Junquera, *J. Heterocycl. Chem.* **1990**, *27*, 269 – 273.

[6] M. Modica, M. Santagati, A. Santagati, V. Cutuli, *et al*., *Pharmazie* **2000**, *55*, 500 – 502.

[7] C. J. Shishoo, K. S. Jain, *J. Heterocycl. Chem.* **1992**, *29*, 883 – 893.

[8] V. Alagarsamy, D. Shankar, V. R. Solomon, *ARKIVOC* **2006**, *16*, 149 – 159.

[9] U. S. Pathak, V. Alagarsamy, *Acta Pharm. Turc.* **1999**, *41*, 37 – 41.

[10] R. K. Russell, J. B. Press, R. A. Rampulla, J. J. McNally, *et al*., *J. Med. Chem.* **1988**, *31*, 1786 – 1793.

[11] U. S. Pathak, S. Singh, J. Padh, *Indian J. Chem.* **1991**, *30B*, 618 – 619.

[12] V. D. Patil, D. S. Wise, L. L. Wotring, L. C. Bloomer, L. B. Townsend, *J. Med. Chem.* **1985**, *28*, 423 – 427.

[13] M. Wiesenfeldt, K. H. Etzbach, P. Hofmeister, C. Künast, K. O. Westphalen (BASF A.G.) *Eur. Pat. Appl.* **1990**, *447891*; *Chem. Abstr.* **1991**, *115*, 256224y.

[14] F. Russo, N. A. Santagati, R. Venturini, S. Spampinato, *Pharmazie* **1990**, *45*, 493 – 495.

[15] M. Sugiyama, T. Sakamoto, K. Tabata, K. Endo, *et al*., *Chem. Pharm. Bull.* (*Tokyo*) **1989**, *37*, 2122 – 2131.

[16] N. L. Shirole, G. S. Talele, R. A. Fursule, S. J. Surana, K. S. Jain, *Asian J. Chem.* **2006**, *18*, 2673 – 2679.

[17] A. Zekany, S. Markleit, *Pharmazie* **1987**, *42*, 160 – 161.

[18] A. A. H. M. Eissa, A. A. Moneer, *Arch. Pharm. Res.* **2004**, *27*, 885 – 892.

[19] S. M. Riyadh, M. A. Abdallah, I. M. Abbas, S. M. Gomha, *Int. J. Pure Appl. Chem.* **2006**, *1*, 57 – 64.

[20] A. E. Rashad, A. H. Shamroukh, M. I. Hegab, H. M. Awad, *Acta Chim. Slov.* **2005**, *52*, 429 – 434.

[21] Y.-G. Hu, A.-H. Zheng, X.-Z. Ruan, M.-W. Ding, *Beilstein J. Org. Chem.* **2008**, *4*, 1 – 6.

[22] W. W. Wardakhan, N. A. Louca, M. M. Kamel, *Acta Chim. Slov.* **2007**, *54*, 229 – 241.

[23] M. Chaykovsky, M. Lin, A. Rosowsky, E. J. Modest, *J. Med. Chem.* **1973**, *16*, 188 – 191.

[24] N. A. Hassan, *J. Sulfur Chem.* **2005**, *26*, 343 – 352.

[25] M. S. Shahabuddin, M. Gopal, S. C. Raghavan, *J. Cancer Mol.* **2007**, *3*, 139 – 146.

[26] M. M. Ghorab, A. N. Osman, E. Noaman, H. I. Heiba, N. H. Zaher, *Phosphorus Sulfur Silicon Relat. Elem.* **2006**, *181*, 1983 – 1996.

[27] M. Modica, G. Romeo, L. Materia, F. Russo, *et al*., *Bioorg. Med. Chem.* **2004**, *12*, 3891 – 3901.

[28] M. M. Ghorab, H. I. Heiba, M. A. El-Gawish, *Phosphorus Sulfur Silicon Relat. Elem.* **1995**, *106*, 85 – 91.

[29] P. K. Swarnkar, P. Kriplani, G. N. Gupta, K. G. Ojha, *J. Chem.* **2007**, *4*, 14 – 20.

[30] A. Nagaraj, C. S. Reddy, *J. Iran. Chem. Soc.* **2008**, *5*, 262 – 267; I. Gil-Ad, B. Shtaif, Y. Levkovitz, J. Nordenberg, *et al*., *Oncol. Rep.* **2006**, *15*, 107 – 112.

[31] Y.-W. Hu, *J. Chin. Chem. Soc.* **2007**, *54*, 1075 – 1085.

[32] A. S. Oganisyan, A. S. Noravyan, A. A. Karapetyan, M. S. Alekanyan, Y. T. Struchkov, *Chem. Heterocycl. Compd.* **2004**, *40*, 79 – 83.

[33] N. A. Danilkina, L. E. Mikhailov, B. A. Ivin, *Russ. J. Org. Chem.* **2006**, *42*, 783 – 814.

[34] U. Vögeli, W. von Philipsborn, K. Nagarajan, M. D. Nair, *Helv. Chim. Acta* **1978**, *61*, 607 – 617.

[35] E. K. Ahmed, U. Sensfuss, W. D. Habicher, *J. Heterocycl. Chem.* **1999**, *36*, 1119 – 1122.

[36] M. B. Hansen, S. E. Nielsen, K. Berg, *J. Immunol. Methods* **1989**, *119*, 203 – 210.

[37] F. T. van Amsterdam, A. Roveri, M. Maiorino, E. Ratti, F. Ursini, *Free Rad. Biol. Med.* **1992**, *12*, 183 – 187.

