Full Paper

Synthesis, Cytotoxicity, and Pro-Apoptosis Activity of Etodolac Hydrazide Derivatives as Anticancer Agents

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Etodolac hydrazide and a novel series of etodolac hydrazide-hydrazones **3–15** and etodolac 4-thiazolidinones **16–26** were synthesized in this study. The structures of the new compounds were determined by spectral (FT-IR, ^{1}H NMR, ^{13}C NMR, HREI-MS) methods. Some selected compounds were determined at one dose toward the full panel of 60 human cancer cell lines by the National Cancer Institute (NCI, Bethesda, USA). 2-(1,8-Diethyl-1,3,4,9-tetrahydropyrano[3,4-*b*]indole-1-yl)acetic acid[(4-chlorophenyl)methylene]hydrazide **9** demonstrated the most marked effect on the prostate cancer cell line PC-3, with 58.24% growth inhibition at 10^{-5} M (10 μM). Using the MTT colorimetric method, compound **9** was evaluated *in vitro* against the prostate cell line PC-3 and the rat fibroblast cell line L-929, for cell viability and growth inhibition at different doses. Compound **9** exhibited anticancer activity with an IC_{50} value of 54 μM (22.842 μg/mL) against the PC-3 cells and did not display any cytotoxicity toward the L-929 rat fibroblasts, compared to etodolac. In addition, this compound was evaluated for caspase-3 and Bcl-2 activation in the apoptosis pathway, which plays a key role in the treatment of cancer.

Keywords: Apoptosis / Etodolac / Hydrazide-hydrazone / PC-3 prostate cancer cell line / 4-Thiazolidinone

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Introduction

Etodolac ((*R*,*S*) 2-[1,8-diethyl-1,3,4-tetrahydrapyrano(3,4-*b*)indole-1-yl]acetic acid) is a nonsteroidal anti-inflammatory drug (NSAID) with analgesic and antipyretic properties. The U.S. Food and Drug Administration approved etodolac for the treatment of inflammation and pain caused by osteoarthritis and rheumatoid arthritis. Etodolac has inhibitory effects on cyclooxygenase-2 (COX-2) activation. Its mechanism of action is the inhibition of COX, leading to a reduction in the synthesis of prostaglandins and other mediators of inflammation and pain. Prostaglandins are thought to play an important role in the proliferation of prostate cancer and are highly expressed in prostate cancer tissues. Epidemiological studies, clinical observations, and animal model studies suggest that NSAIDs have the potential to protect against carcinogenesis due to their inhibitory effect on COX-2 [1–3]. In recent years, anti-cancer effects of NSAIDs through a COX-2-independent mechanism have been revealed. COX-2 inhibitors such as etodolac suppress proliferation, induce apoptosis in prostate cancer cells and have no effect on normal prostate stromal cells [4, 5]. Recently, it has

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been reported that etodolac has antitumor activity on many types of cancer, such as urogenital system cancers [4, 6], Burkitt's lymphoma [7], multiple myeloma, chronic lymphocytic leukemia, and prostate cancer [8–10].

Hydrazones possessing an azomethine (–NHN=CH–) proton constitute an important class of compounds for new drug development. Therefore, many researchers have synthesized these compounds as target structures and evaluated their biological activities [11]. The search for more effective and less toxic anticancer drugs led to the discovery of hydrazide-hydrazones including new compounds having anticancer activity [12]. In previous studies, it has been reported that hydrazide-hydrazones possess antitumoral activity [13–20]. There is also a report on the discovery of a hydrazone derivative as an apoptosis inducer that caused a high degree of growth inhibition in A549 lung cancer cells [21].

Another important biological scaffold is given by 4-thiazolidinones, which are used in the field of medicinal chemistry and possess a wide range of promising biological activities [22]. The 4-thiazolidinone derivatives have demonstrated significant anticancer activity [23–27].

These facts and our interest in the synthesis of newer anticancer compounds prompted us to undertake the synthesis of novel hydrazide-hydrazones and their subsequent conversion into 4-thiazolidinone derivatives starting from etodolac and to evaluate their anticancer potential. These observations guided us in the development of new etodolac hydrazide-hydrazones and 4-thiazolidinones that might possess various biological activities.

Results

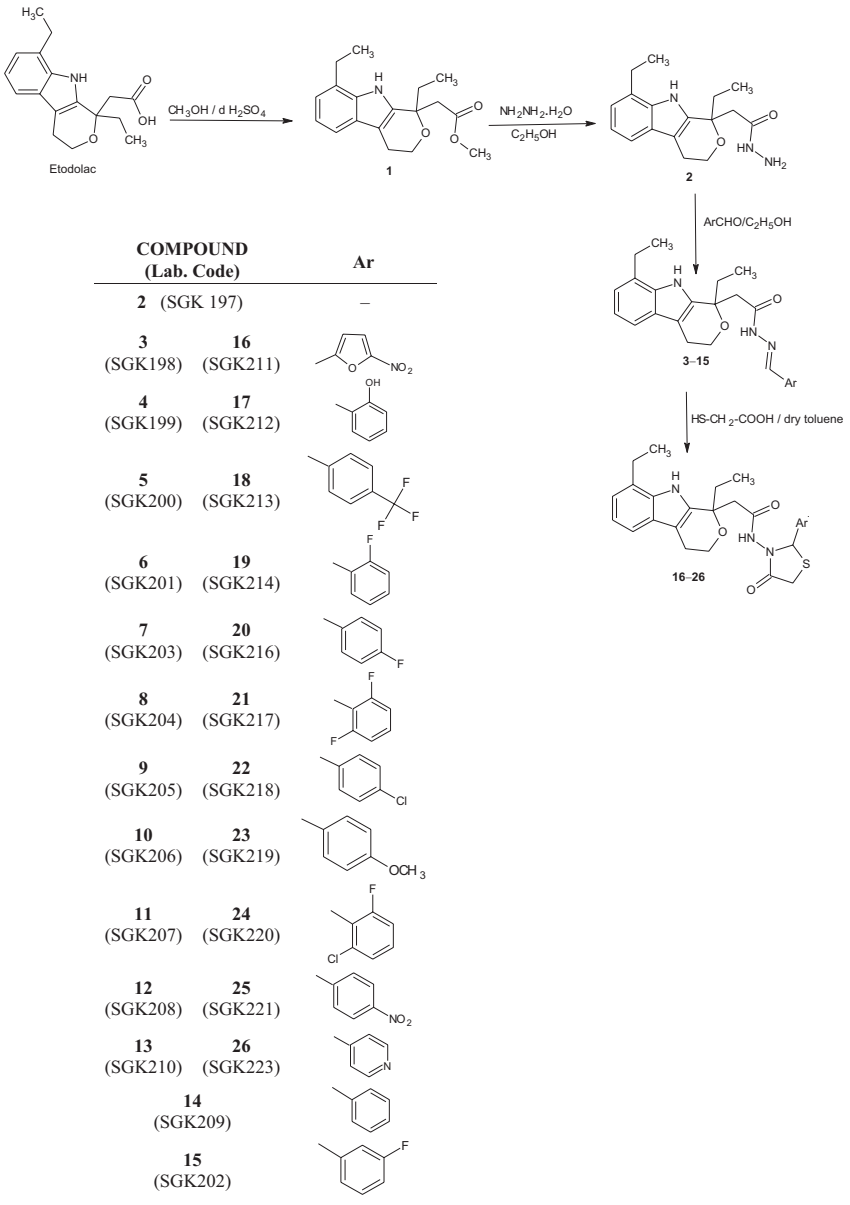
Chemistry

(*R*,*S*) 2-[1,8-Diethyl-1,3,4-tetrahydrapyrano(3,4-*b*)indole-1-yl]acetic acid (etodolac), which possesses analgesic, antipyretic, and anti-inflammatory activities, was chosen as the starting compound to design hydrazide-hydrazone and 4-thiazolidinone derivatives (Scheme 1). Methyl(1,8-diethyl-1,3,4,9-tetrahydropyrano[3,4-*b*]indole-1-yl)acetate **1** was synthesized by the reaction of etodolac and methanol in the presence of a few drops of concentrated sulfuric acid. By heating this methyl ester with hydrazine-hydrate in methanol, we obtained 2-(1,8-diethyl-1,3,4,9-tetrahydropyrano[3,4-*b*]indole-1-yl)acetohydrazide **2**. After condensing hydrazide **2** with aromatic aldehydes in ethanol, 2-(1,8-diethyl-1,3,4,9-tetrahydropyrano[3,4-*b*]indole-1-yl)acetic acid[(5-nitro-2-furyl/substituted phenyl)methylene]hydrazides **3–15** were obtained in good yields (59–98%). 3-(2-(1,8-Diethyl-1,3,4,9-tetrahydropyrano[3,4-*b*]indol-1-yl)acetylhydrazono)-2-alkyl/aryl-4-thiazolidinones **16–26** were obtained in moderate yields (15–98%) by refluxing the related etodolac hydrazide-hydrazones **3–15** and thioglycolic acid in dry toluene for 9–16 h using a Dean–Stark water separator. Purification of the synthesized compounds in this study was confirmed by thin-layer chromatography (TLC) and microanalysis. The structures of these compounds synthesized from etodolac were identified by the help of elemental analysis and FT-IR, ^{1}H NMR, and HR-MS (compounds **16–26**) spectral data.

Etodolac methyl ester has previously been reported in the literature [28, 29]; however, we synthesized this compound using a different method in this study. Etodolac hydrazide **2** was first reported in this study. In the ^{1}H NMR spectrum of 2-(1,8-diethyl-1,3,4,9-tetrahydropyrano[3,4-*b*]indole-1-yl)acetohydrazide **2**, singlet signals derived from the hydrazide structure appeared at 4.27 ppm (–NHN$\underline{H}_2$) and 8.94 ppm (–N$\underline{H}$NH$_2$), showing the integration of two protons and one proton, respectively.

Etodolac hydrazide-hydrazones were characterized by IR spectra displaying C=O bands at 1639–1667 cm^{-1} and C=N bands at 1601–1630 cm^{-1} [19, 30]. The ^{1}H NMR data were also in agreement with the formation of hydrazide-hydrazone. The signals appearing at 7.92–8.46 ppm in the ^{1}H NMR spectra were attributed to the –N=CH proton, and the proton loss at 4.27 ppm was attributed to –CO–NH–N$\underline{H}_2$ [31]. According to the literature, the hydrazones may exist as *E*/*Z* geometrical isomers around C=N double bonds and as *cis*/*trans* amide conformers [32]. The ^{1}H NMR spectra of compounds **3–15** displayed the resonance of a hydrazone N–H at 11.04–11.55 ppm in the *Z* isomer and at 11.21–11.74 ppm in the *E* isomer. Azomethine protons of the compounds resonated at 7.92–8.30 ppm in the *E* isomer and at 8.15–8.46 ppm in the *Z* isomer in dimethyl sulfoxide-d_6 (DMSO-d_6) solution. The percentage of each isomer was calculated using the integral values of the peak pairs and the dominating isomer was assigned to the *Z* isomer due to azomethine protons. Also, –CH$_3$ protons at the C$_1$ position were observed as two triplets due to the canonic form. In addition, the NH protons of compound **10** were observed to exchange with D$_2$O in the spectrum. All the other aliphatic and aromatic protons were observed in the expected regions. The heteronuclear multiple bond correlation (HMBC) spectrum of compound **9** also confirmed the detection of long-range ^{1}H–^{13}C couplings. The structure of hydrazide-hydrazone **9** was established by using an HMBC experiment as shown in Fig. 1.

4-Thiazolidinones **16–26** were prepared by cyclization of the etodolac hydrazide-hydrazones **3–15** with thioglycolic acid. The reaction proceeded by nucleophilic attack of the anion of thioglycolic acid upon the carbon atom of a Schiff base, followed by the capture of a proton by nitrogen and cyclization of the latter with removal of a water molecule, using a Dean–Stark water separator. Formation of 4-thiazolidinones **16–26** was confirmed by IR spectroscopy, which showed the ring C=O stretching characteristic of lactam in the range of 1705–1722 cm^{-1} and amide C=O bands at



COMPOUND (Lab. Code)		Ar
2 (SGK 197)		–
3 (SGK198)	**16** (SGK211)	
4 (SGK199)	**17** (SGK212)	
5 (SGK200)	**18** (SGK213)	
6 (SGK201)	**19** (SGK214)	
7 (SGK203)	**20** (SGK216)	
8 (SGK204)	**21** (SGK217)	
9 (SGK205)	**22** (SGK218)	
10 (SGK206)	**23** (SGK219)	
11 (SGK207)	**24** (SGK220)	
12 (SGK208)	**25** (SGK221)	
13 (SGK210)	**26** (SGK223)	
14 (SGK209)		
15 (SGK202)		

Scheme 1. Synthetic route of compounds **1–26**.

1653–1682 cm^{-1} [33]. The structures of compounds **16–26** were supported by IR spectra as observed in **3–15** with the disappearance of 1601–1630 cm^{-1} for the –N=CH band and 1705–1722 cm^{-1} for the C=O of thiazolidinone [34–37]. The ^1H NMR and ^{13}C NMR data were also in agreement with the formation of the 4-thiazolidinone ring. The ^1H NMR spectra of compounds **16–26** lack the CH=N signal at 7.92–8.46 ppm, providing confirmatory evidence for the ring closure from hydrazones **3–15**. The ^1H NMR spectra of the 4-thiazolidinones displayed resonances assigned to the heterocyclic methylene ring protons at 3.66–3.94 and 3.82–4.12 ppm, corresponding to two diastereotopic protons. Due to magnetic nonequivalance caused by the chiral center produced by the nucleophilic addition of thioglycolic acid to the N=CH function of etodolac hydrazide-hydrazones, the methylene groups of etodolac 4-thiazolidinones appeared as two doublets with coupling constants characteristic for geminal protons (J = 14.2–17.7 Hz). Additional support for the structure of the 4-thiazolidinones was obtained from the absorption positions of the N–C<u>H</u>–S protons, which showed upfield shifts and resonated at about 5.13–6.41 ppm due to the loss of the sp^2 character of the involved C-atom [38–41]. The appearance of the C_5–H and C_2–H protons, characteristic of thiazolidinones, confirmed the addition of thioglycolic

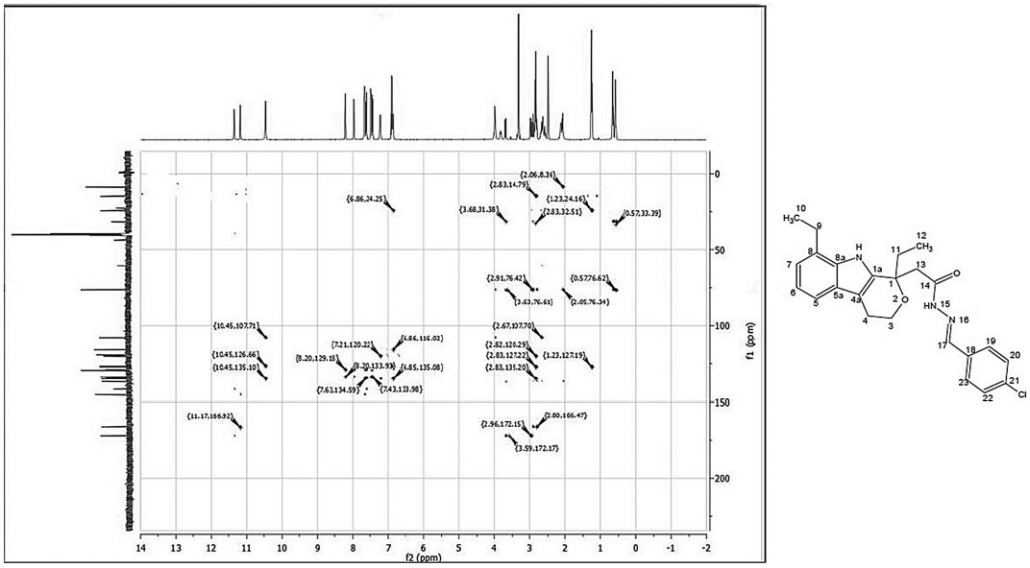


Figure 1. HMBC spectrum of compound **9**.

acid across the azomethine linkage in the compounds. The chemical shifts of the aromatic and NH protons of the compounds have the expected values. The ^{13}C NMR spectra of the compounds **17**, **18**, **20**, **21**, **23**, **25**, and **26**, which were chosen as prototypes, verified the proposed 4-thiazolidinone structure [34, 41]. The peaks resonated at 30.95–37.07, 60.90–65.92, 163.00–171.4, and 169.34–172.00 ppm for S–$\underline{C}H_2$, S–$\underline{C}H$–N, amide C=O, and thiazolidinone C=O, respectively, in the ^{13}C NMR spectra of the 4-thiazolidinones. High-resolution mass spectra (HR-MS) confirmed the molecular weights and empirical formulae of the compounds and fragments, with less than 5 mmu bias between the calculated and experimental *m/z* values of either the molecular or the fragment ions. The ionization mode was electron impact (EI) for all 4-thiazolidinone derivatives. The fragmentation pattern for all etodolac 4-thiazolidinones (**16**–**26**) supported the proposed structures. The fragment ion peaks, especially of $[M-CH_2S]^+$, $[M-COCH_2S]^+$, and $[(M+1)-S]^+$, are the diagnostic peaks for thiazolidinone rings [34, 42].

Biological activity

The primary anticancer assay was performed in accordance with the protocol of the Drug Evaluation Branch, National Cancer Institute (NCI), Bethesda [43–45]. As most of the compounds in the submitted series of structures include one or more functional groups that have been found troublesome in the development of successful drug candidates, compounds **1**, **9**, **10**, **20**, and **21** were chosen as prototypes and evaluated against the full panel of 60 human tumor cell lines at a single dose for screening of their anticancer potential. In addition, the selection criteria guidance is available online at the DTP web site (www.dtp.nci.nih.gov/docs/misc/common_files/guidelines.html). The anticancer screening data at a concentration of 10^{-5} M are summarized in Fig. 2. In general, compound **9** displayed antiproliferative activity against three cancer cell lines (renal cancer, UO31; prostate cancer, PC-3; non-small-cell lung cancer, HOP-92). The most potent derivative was identified as 2-(1,8-diethyl-1,3,4,9-tetrahydropyrano[3,4-*b*]indole-1-yl)acetic acid[(4-chlorophenyl)methylene]hydrazide (compound **9**), which demonstrated the most marked effect on the prostate cancer cell line PC-3 (cell growth promotion 41.76%, inhibition 58.24%). However, compound **1**, the starting compound for synthesis, showed lower activity against the PC-3 cell line. Moreover, compound **9** demostrated strong cytotoxicity against the melanoma cell line SK-MEL-28.

The cell viability and growth inhibition (L-929 and PC-3) was studied at the Marmara University Faculty of Pharmacy,

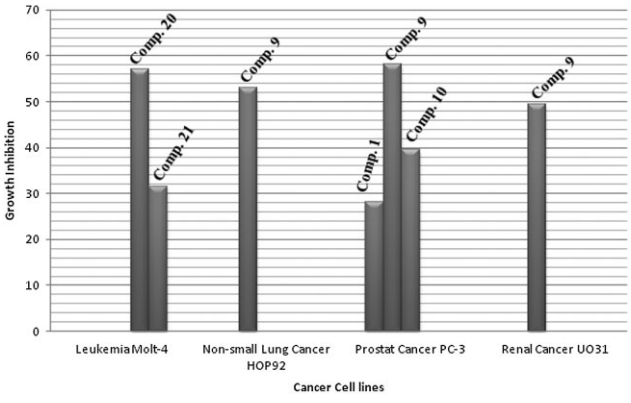


Figure 2. Growth inhibition data of compounds **1**, **9**, **10**, **20**, and **21** at 10^{-5} M concentration.

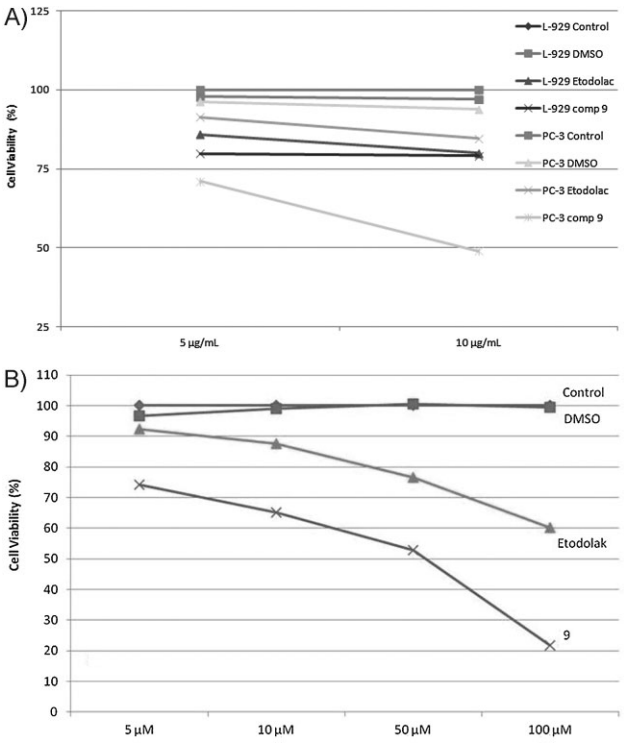


Figure 3. (A) Effects of etodolac and compound **9** on the viability of L-929 and PC-3 cells. (B) Effects of compound **9** on the viability of PC-3 cancer cells.

Department of Pharmaceutical Biotechnology. On the basis of the above results, the cell viability assessment was performed by measuring the levels of live cells after incubation with different doses of etodolac and compound **9** for 48 h, using the MTT colorimetric assay against a panel of L-929 rat fibroblast cell (ATCC, CCL-1) and PC-3 prostate cancer cell (ATCC, CRL-1435) lines (100 indicates no activity; 0 indicates complete cell death) at a final DMSO concentration of 0.1–0.05%. The cell viability results are presented in Fig. 3.

When compared to etodolac, which was used as standard, compound **9** did not cause a significant effect on the viability of L-929 rat fibroblasts at the applied doses. This clearly indicates that the hydrazide-hydrazone structure of compound **9** does not have higher toxic effects toward L-929 rat fibroblasts. Compound **9** also gave rise to a significant change in cell viability in the PC-3 prostate cancer cell line at different doses, compared to the standard drug etodolac. Compound **9**, having a hydrazide-hydrazone moiety, was responsible for a significant decrease in cell viability compared to etodolac. The growth inhibitory property (IC_{50}) for compound **9** was 54 μM (22.842 μg/mL).

In the present study, we aimed at investigating whether the influence of compound **9** and etodolac occurred via the apoptotic pathway and, if so, which apoptotic pathway was involved in this process. This part of the study was performed at the Department of Biochemistry, Faculty of Pharmacy, Marmara University. Apoptosis is one of the major mechanisms of cell death in response to cancer therapies. Signaling for apoptosis occurs through multiple independent pathways that are initiated by diverse extracellular and intracellular factors. The intrinsic cell death pathway is also known as the mitochondrial apoptotic pathway [46]. It is known that many apoptotic biochemical processes are regulated by biochemical signaling agents found within mitochondria. Mitochondria also play a central role in the integration and propagation of death signals originating from inside the cell, such as DNA damage and oxidative stress [47, 48]. In this study, the effects of etodolac and compound **9** on apoptosis were investigated via determination of the depolarization of the mitochondrial membrane potential and by assessing the Bcl-2 and caspase-3 levels. As shown in Fig. 4, after the cells were treated with etodolac and compound **9**, the depolarization of the mitochondrial membrane potential increased as compared with the control group ($p < 0.01$) (a colored version of Fig. 4 is provided as Supporting Information Figure S1). At the same time, compound **9** significantly increased the depolarization of the mitochondrial membrane potential when compared with etodolac. Figure 4 shows the alterations of the mitochondrial membrane potential in PC-3 cells after treatment with etodolac and compound **9**. The mitochondrial membrane potential changes were determined using the J-aggregate-forming lipophilic cationic fluorochrome 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazole carbocyanine iodide (JC–1). The dye accumulates and aggregates in mitochondria, emitting a bright red fluorescence. In apoptotic and necrotic cells with altered mitochondrial membrane potential, the dye remains in the cytoplasm in its monomeric form and the fluorescence is green. Loss of mitochondrial potential was indicated by increased green fluorescence. Etodolac and compound **9** increased the loss of mitochondrial potential as compared with the control group. The Bcl-2 levels of the etodolac and compound **9** groups were significantly lower than those of the control group ($p < 0.01$; Fig. 5). There were no significant differences between etodolac and compound **9** in terms of the Bcl-2 levels. The caspase-3 levels are shown in Fig. 6. Etodolac and compound **9** caused a significant increase in the caspase-3 activity as compared with the control group ($p < 0.05$ and 0.01, respectively). There were no significant differences between etodolac and compound **9** in terms of the caspase-3 levels.

Loss of mitochondrial membrane potential and release of mitochondrial proteins are of central importance in mediating and enhancing apoptotic pathways. In this study, we investigated the effects of etodolac and compound **9** on the mitochondrial membrane potential and Bcl-2 as mitochon-

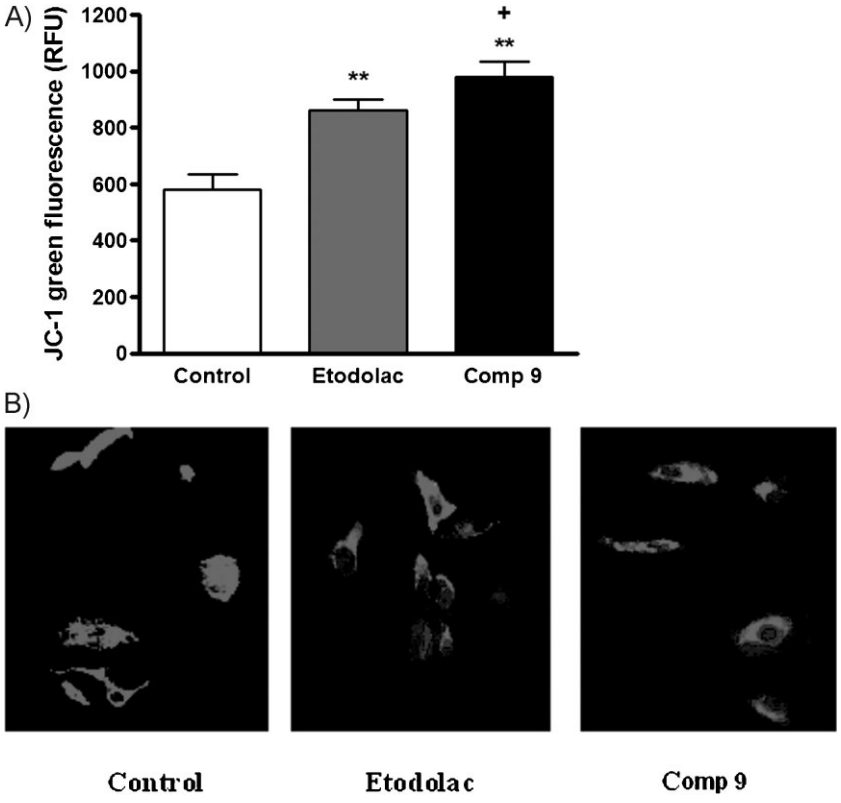


Figure 4. (A) Effects of etodolac and compound **9** on the mitochondrial membrane potential in PC-3 cells ($^{**}p < 0.01$ vs. control, $^{+}p < 0.05$ vs. etodolac). (B) Mean values of JC-1 green fluorescence for etodolac and compound **9**, indicating the mitochondrial membrane potential in the PC-3 cell line. (Fluorescence microscopy after JC-1 staining showing changes in the mitochondrial membrane potential in PC-3 cells; magnification: ×200.)

drial apoptotic markers and on caspase-3 activation as marker for the point of convergence of the intrinsic and extrinsic apoptotic pathways in the PC-3 cell line. Our data show that 24-h treatment with etodolac and compound **9** directly down-regulated Bcl-2 expression and induced caspase-3-dependent apoptosis in the PC-3 cell line. Additionally, these agents caused mitochondrial membrane potential loss in the PC-3 cells. It has been shown that etodolac induced a disturbance of the mitochondrial membrane potential and caused caspase-9, -7, and -3 activation in addition to the reduction of Bcl-2 levels in myeloma cells [49]. The central function of mammalian Bcl-2 family members is to guard the mitochondrial integrity and to control the release of mitochondrial proteins into the cytoplasm [50]. Bcl-2, an anti-apoptotic protein, prevents the release of cytochrome *c* from mitochondria and the activation of caspases, resulting in anti-apoptotic action and the maintenance of cell survival [51]. Our findings indicate that etodolac and

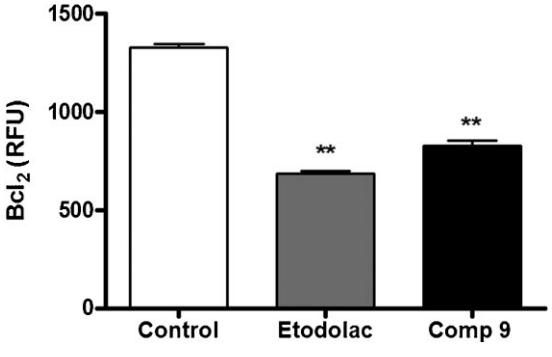


Figure 5. Effects of etodolac and compound **9** on Bcl-2 in PC-3 cells ($^{**}p < 0.01$ vs. control).

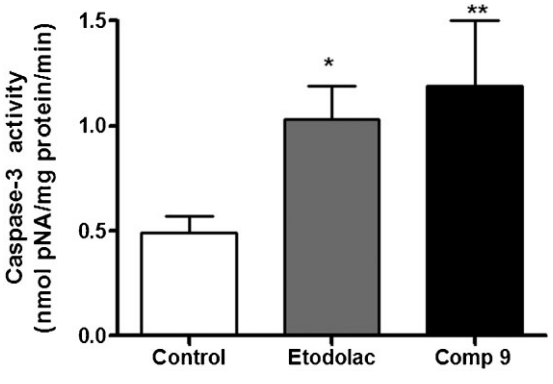


Figure 6. Effects of etodolac and compound **9** on caspase-3 activity in PC-3 cells ($^{*}p < 0.05$ vs. control, $^{**}p < 0.01$ vs. control).

compound **9** induce apoptosis in human PC-3 prostate cancer cells through the mitochondrial pathway.

Discussion

In this study, etodolac hydrazide and a series of novel etodolac hydrazide derivatives were synthesized and evaluated for their anticancer activity. After a preliminary survey, compound **9** was selected by the NCI for anticancer screening. This compound was found to show significant inhibition on the prostate cancer cell line PC-3.

In this study, we identified compound **9** as a promising lead compound for anticancer drug development. This study indicates that the synthesis of hydrazone and 4-thiazolidinone derivatives of etodolac resulted in more effective compounds compared to the starting compound, etodolac. Based on these results, we are now in the process of synthesizing modified analogs of the lead compound in order to generate more effective anticancer agents. The results from the anticancer and apoptosis studies on compound **9**, which was synthesized from racemic etodolac, indicate the need for an evaluation of the above-mentioned activities of the *S*- and *R*-isomers of hydrazones. Thus, the apoptosis results obtained from this planned study will provide an explanation of the apoptotic pathway based on enantiomeric products.

Experimental

Chemistry

All chemicals were purchased from Merck, Sigma–Aldrich, or Fluka. Reactions were monitored by TLC on silica gel plates purchased from Merck. Melting points of the synthesized compounds were determined in a Schmelzpunktgerät SMP II melting point apparatus and are uncorrected. The purity of the compounds was checked on TLC plates precoated with silica gel G using the solvent systems M_1 (petroleum ether/acetone 60:40 v/v), M_2 (petroleum ether/ethyl acetate 60:40 v/v), M_3 (petroleum ether/ethyl acetate 50:50 v/v), M_4 (petroleum ether/ethyl acetate 70:30 v/v), M_5 (petroleum ether/ethyl acetate 30:70 v/v), M_6 (chloroform/methanol 80:20 v/v), and M_7 (chloroform/methanol 60:40 v/v). The spots were located under UV light (254 nm) ($T = 21^{\circ}C$). Elemental analyses were performed on a VarioMICRO V1.5.7. instrument. FT-IR spectra were recorded on a Shimadzu FT-IR-8400S spectrophotometer. ^{1}H NMR spectra were recorded on Bruker Avance-DPX 400 (400 MHz), Bruker Avance 500 (500 MHz), and Varian NMR Mercury 300 (300 MHz) NMR spectrometers using DMSO-d_6 as solvent. MALDI-TOF HR-MS spectra using the EI and FAB ionization techniques were performed using a Jeol JMS-700 instrument. Mass spectra (MS) were determined on an Agilent 1100 LC-MS mass spectrometer. Chemical shifts (δ) are reported in parts per million (ppm). Data are reported as follows: chemical shift, multiplicity (b.s.: broad singlet, d: doublet, m: multiplet, s: singlet, and t: triplet), coupling constants (Hz), integration. ^{13}C NMR spectra were recorded on Bruker Avance-DPX 100 (100 MHz) and Bruker Avance-75 (75 MHz) spectrometers.

*Methyl (1,8-diethyl-1,3,4,9-tetrahydropyrano[3,4-b]indole-1-yl)acetate (**1**)*

Etodolac (0.01 mol) and methanol (16 mL) were refluxed for 3 h in a few drops of concentrated sulfuric acid. The contents of the flask were subsequently cooled and neutralized by using $NaHCO_3$ (5%). The resulting precipitate was filtered, dried, and recrystallized twice from ethanol. Cream-colored solid. MW: 301.380. m.p. 128–130°C [28, 29]. Yield 66%. Rf × 100 value 87 (M_1). IR (ν_{max}, cm^{-1}): 3379 (indole NH), 1705 (C=O). ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 0.83 (3H, t, $-CH_2-CH_3$ at C_1); 1.37 (3H, t, $-CH_2-CH_3$ at C_8); 1.98–2.18 (2H, m, $-CH_2-CH_3$ at C_1); 2.73–3.04 (6H, m, $-CH_2-CH_3$ at C_8, $-CH_2-COOCH_3$ at C_1 and $-CH_2$ at C_4); 3.72 (3H, s, $-COOCH_3$); 3.94–4.06 (2H, m, $-CH_2$ at C_3); 7.01 (1H, d, C_7–H, $J = 6.8$ Hz); 7.06 (1H, t, C_6–H); 7.36 (1H, d, C_5–H, $J = 7.2$ Hz); 9.06 (s, 1H, indole N–H). Anal. calcd. for $C_{18}H_{23}NO_3$: C, 71.73; H, 7.69; N, 4.65%. Found: C, 70.85; H, 7.24; N, 4.75%.

*2-(1,8-Diethyl-1,3,4,9-tetrahydropyrano[3,4-b]indole-1-yl)acetohydrazide (**2**)*

To a methanolic solution of compound **1** (20 mL, 0.01 mol) was added hydrazine-hydrate (80%, 7 mL) and refluxed for 3 h. The reaction mixture was then cooled, diluted with water and allowed to stand overnight. The precipitated solid was washed with water, dried, and recrystallized twice from petroleum ether.

White colored solid. MW: 301.383. m.p. 186–188°C. Yield 80%. Rf × 100 value 51 (M_1). FT-IR (ν_{max}, cm^{-1}): 3354, 3310 (indole and hydrazide NH), 1651 (C=O). ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 0.62 (3H, t, $-CH_2-CH_3$ at C_1); 1.26 (3H, t, $-CH_2-CH_3$ at C_8); 2.00–2.07 (2H, m, $-CH_2-CH_3$ at C_1); 2.58–2.86 (6H, m, $CH_2-CONHNH_2$, $-CH_2-CH_3$ at C_8 and $-CH_2$ at C_4); 3.91–3.97 (2H, m, $-CH_2$ at C_3); 4.27 (2H, b.s., NH–NH_2); 6.87–7.24 (3H, m, Ar–H); 8.94 (1H, s, $NHNH_2$); 10.55 (s, 1H, indole N–H). Anal. calcd. for $C_{17}H_{23}N_3O_2$: C, 67.75; H, 7.69; N, 13.94%. Found: C, 67.33; H, 6.95; N, 13.73%.

*General procedure for the synthesis of 2-(1,8-diethyl-1,3,4,9-tetrahydropyrano[3,4-b]indole-1-yl)acetic acid[(5-nitro-2-furyl/substituted phenyl)methylene]hydrazides **3–15***

A solution of compound **2** (0.01 mol) and equimolar amounts of appropriate aromatic aldehyde in absolute ethanol (30 mL) was heated under reflux for 3 h. The obtained precipitate was filtered off, dried, and recrystallized twice from ethanol.

*2-(1,8-Diethyl-1,3,4,9-tetrahydropyrano[3,4-b]indole-1-yl)acetic acid[(5-nitro-2-furyl)methylene]hydrazide (**3**)*

Dark orange solid. MW: 425.450. m.p. 165–167°C. Yield 96%. Rf × 100 value: 22 (M_2). FT-IR (ν_{max}, cm^{-1}): 3449, 3349, 3214 (indole and hydrazone NH), 1653 (C=O), 1607 (C=N). ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 0.58, 0.67 (3H, tt, $-CH_2-CH_3$ at C_1); 1.18–1.28 (3H, m, $-CH_2-CH_3$ at C_8); 2.04–2.13 (2H, m, $-CH_2-CH_3$ at C_1); 2.61–2.69 (2H, m, $-CH_2-CH_3$ at C_8); 2.83–3.72 (m, 4H, $-CH_2-CONH$ and $-CH_2$ at C_4); 3.81–4.09 (2H, m, $-CH_2$ at C_3); 6.87–7.78 (5H, m, Ar–H); 7.92, 8.24 (1H, ss, –N=CH); 10.51 (1H, d, indole N–H); 11.55, 11.74 (1H, ss, –CO–NH). Anal. calcd. for $C_{22}H_{24}N_4O_5$: C, 62.25; H, 5.70; N, 13.20%. Found: C, 62.12; H, 5.71; N, 12.95%.

*2-(1,8-Diethyl-1,3,4,9-tetrahydropyrano[3,4-b]indole-1-yl)acetic acid[(2-hydroxy phenyl)methylene]hydrazide (**4**)*

Light cream-colored solid. MW: 405.490. m.p. 160°C. Yield 79%. Rf × 100 value: 29.51 (M_2). FT-IR (ν_{max}, cm^{-1}): 3379, 3264 (indole and hydrazone NH, OH), 1667 (C=O), 1618 (C=N). ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 0.60–0.68 (3H, m, $-CH_2-CH_3$ at C_1); 1.24–1.29 (3H, m, $-CH_2-CH_3$ at C_8); 2.07–2.12 (2H, m, $-CH_2-CH_3$ at C_1); 2.62–2.70 (2H, m, $-CH_2-CH_3$ at C_8); 2.82–2.96 (4H, m, $-CH_2CONH$ and $-CH_2$ at C_4); 3.54–4.01 (2H, m, $-CH_2$ at C_3); 6.88–7.58 (7H, m, Ar–H); 8.30, 8.42 (1H, ss, N=CH); 10.08, 10.51 (1H, ss, indole N–H); 11.18, 11.29 (1H, s, CO–NH); 11.42 (1H, s, –OH). Anal. calcd. for $C_{24}H_{27}N_3O_3$: C, 71.09; H, 6.71; N, 10.36%. Found: C, 70.98; H, 6.76; N, 10.21%.

*2-(1,8-Diethyl-1,3,4,9-tetrahydropyrano[3,4-b]indole-1-yl)-acetic acid[(4-trifluoromethylphenyl)methylene]-hydrazide (**5**)*

Cream-colored solid. MW: 466.496. m.p. 140°C. Yield 86%. Rf × 100 value: 41.93 (M_2). FT-IR (ν_{max}, cm^{-1}): 3424, 3358, 3312 (indole and hydrazone NH), 1651 (C=O), 1622 (C=N). ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 0.60, 0.67 (3H, tt, $-CH_2-CH_3$ at C_1); 1.26 (3H, t, $-CH_2-CH_3$ at C_8); 2.02–2.10 (2H, m, $-CH_2-CH_3$ at C_1); 2.54–2.66 (2H, m, $-CH_2-CH_3$ at C_8); 2.83–3.01 (4H, m, $-CH_2CONH-$ at C_1 and $-CH_2$ at C_4); 3.56–3.92 (2H, m, $-CH_2$ at C_3); 6.89–7.90 (7H, m, Ar–H); 8.06, 8.30 (1H, ss, –N=CH); 10.51 (d, 1H, indole N–H); 11.36, 11.53 (1H, ss, –CO–NH–). Anal. calcd. for $C_{25}H_{26}F_3N_3O_2.1/2H_2O$: C, 64.37; H, 5.83; N, 9.01%. Found: C, 64.87; H, 5.19; N, 8.37%.

*2-(1,8-Diethyl-1,3,4,9-tetrahydropyrano[3,4-b]indole-1-yl)acetic acid[(2-fluorophenyl)methylene]hydrazide (**6**)*

Light cream-colored solid. MW: 407.480. m.p. 138°C. Yield 65%. Rf × 100 value: 50.82 (M_2). FT-IR (ν_{max}, cm^{-1}): 3302 (indole and hydrazone NH), 1645 (C=O), 1605 (C=N). ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 0.59, 0.66 (3H, tt, $-CH_2-CH_3$ at C_1); 1.24–1.28 (3H, m, $-CH_2-CH_3$ at C_8); 2.08–2.14 (2H, m, $-CH_2-CH_3$ at C_1); 2.60–2.69 (2H, m, $-CH_2-CH_3$ at C_8); 2.81–3.55 (4H, m, $-CH_2CONH$ at C_1 and $-CH_2$ at C_4); 3.69–4.01 (2H, m, $-CH_2$ at C_3); 6.87–7.89 (7H, m, Ar–H); 8.21, 8.46 (1H, ss, –N=CH); 10.51 (1H, s, indole N–H); 11.33, 11.46 (1H, ss, –CO–NH). Anal. calcd. for $C_{24}H_{26}FN_3O_2$: C, 70.74; H, 6.43; N, 10.31%. Found: C, 70.35; H, 5.84; N, 10.17%.

*2-(1,8-Diethyl-1,3,4,9-tetrahydropyrano[3,4-b]indole-1-yl)acetic acid[(4-fluorophenyl)methylene]hydrazide (**7**)*

Cream-colored solid. MW: 407.480. m.p. 148°C. Yield 98%. Rf × 100 value: 31.15 (M_2). FT-IR (ν_{max}, cm^{-1}): 3379, 3239 (indole and hydrazone NH), 1661 (C=O), 1601 (C=N). ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 0.59, 0.66 (3H, tt, $-CH_2-CH_3$ at C_1); 1.24–1.28 (3H, m, $-CH_2-CH_3$ at C_8); 2.05–2.15 (2H, m, $-CH_2-CH_3$ at C_1); 2.60–2.69 (2H, m, $-CH_2-CH_3$ at C_8); 2.80–3.56 (4H, m, $-CH_2CONH$ at C_1 and $-CH_2$ at C_4); 3.69–4.01 (2H, m, $-CH_2$ at C_3); 6.87–7.75 (7H, m, Ar–H); 7.99, 8.22 (1H, ss, –N=CH–); 10.51 (1H, d, indole N–H); 11.17, 11.34 (1H, ss, –CO–NH). Anal. calcd. for $C_{24}H_{26}FN_3O_2$: C, 70.74; H, 6.43; N, 10.31%. Found: C, 70.58; H, 6.39; N, 9.92%.

*2-(1,8-Diethyl-1,3,4,9-tetrahydropyrano[3,4-b]indole-1-yl)acetic acid[(2,6-difluorophenyl)methylene]hydrazide (**8**)*

Light brown solid. MW: 425.471. m.p. 145°C. Yield 94%. Rf × 100 value: 47.54 (M_2). FT-IR (ν_{max}, cm^{-1}): 3323 (indole and hydrazone NH), 1645 (C=O), 1624 (C=N). ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 0.59, 0.66 (3H, tt, $-CH_2-CH_3$ at C_1); 1.24–1.29 (3H, m, $-CH_2-CH_3$ at C_8); 2.06–2.19 (2H, m, $-CH_2-CH_3$ at C_1); 2.56–2.70 (2H, m, $-CH_2-CH_3$ at C_8); 2.81–3.71 (4H, m, $-CH_2CONH$ at C_1 and $-CH_2$ at C_4); 3.82–4.03 (2H, m, $-CH_2$ at C_3); 6.87–7.52 (6H, m, Ar–H); 8.13, 8.38 (1H, ss, –N=CH); 10.47 (1H, d, indole N–H); 11.33, 11.47 (1H, ss, –CO–NH). Anal. calcd. for $C_{24}H_{25}F_2N_3O_2$: C, 67.75; H, 5.92; N, 9.88%. Found: C, 67.67; H, 5.90; N, 9.69%.

*2-(1,8-Diethyl-1,3,4,9-tetrahydropyrano[3,4-b]indole-1-yl)acetic acid[(4-chlorophenyl)methylene]hydrazide (**9**)*

White solid. MW: 423.935. m.p. 174°C. Yield 80%. Rf × 100 value: 36.07 (M_2). FT-IR (ν_{max}, cm^{-1}): 3231, 3213 (indole and hydrazone NH), 1659 (C=O), 1612 (C=N). ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 0.59, 0.66 (3H, tt, $-CH_2-CH_3$ at C_1); 1.24–1.28 (3H, m, $-CH_2-CH_3$ at C_8); 2.07–2.15 (2H, m, $-CH_2-CH_3$ at C_1); 2.60–2.69 (2H, m, $-CH_2-CH_3$ at C_8); 2.81–2.98 (m, 4H, $-CH_2CONH$ at C_1 and $-CH_2$ at C_4); 3.70–4.01 (2H, m, $-CH_2$ at C_3); 6.87–7.71 (7H, m, Ar–H); 7.98, 8.22 (1H, ss, –N=CH–); 10.51 (1H, d, indole N–H); 11.23, 11.39 (1H, ss, –CO–NH). Anal. calcd. for $C_{24}H_{26}ClN_3O_2$: C, 68.00; H, 6.18; N, 9.91%. Found: C, 68.14; H, 6.27; N, 9.89%.

*2-(1,8-Diethyl-1,3,4,9-tetrahydropyrano[3,4-b]indole-1-yl)acetic acid[(4-methoxyphenyl)methylene]acetohydrazide (**10**)*

White solid. MW: 419.516. m.p. 171–173°C. Yield 76%. Rf × 100 value: 44.44 (M_3). FT-IR (ν_{max}, cm^{-1}): 3297, 3260 (indole and hydrazone NH), 1647 (C=O), 1605 (C=N). ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 0.59, 0.66 (3H, tt, $-CH_2-CH_3$ at C_1); 1.06–1.29 (3H, m, $-CH_2-CH_3$ at C_8); 2.07–2.16 (2H, m, $-CH_2-CH_3$ at C_1); 2.60–2.70 (m, 2H, CH_2-CH_3 at C_8); 2.78–3.71 (m, 4H, $-CH_2CONH$ at C_1 and $-CH_2$ at C_4); 3.80 (3H, s, $-OCH_3$); 3.84–4.01 (2H, m, $-CH_2$ at C_3); 6.85–7.63 (7H, m, Ar–H); 7.94, 8.15 (1H, ss, N=CH); 10.51 (1H, d, indole N–H); 11.04, 11.21 (1H, ss, –CO–NH). Anal. calcd. for $C_{25}H_{29}N_3O_3$: C, 71.57; H, 6.97; N, 10.02%. Found: C, 71.35; H, 6.84; N, 9. 91%. MS [API-ES, *m/z* (%)]: 420.3 ($[M^+]^{+1}$, 4.2), 419.3 (25.7), 418.2 (100), 225.5 (8.8), 169.3 (2.2).

*2-(1,8-Diethyl-1,3,4,9-tetrahydropyrano[3,4-b]indole-1-yl)acetic acid[(2-chloro-6-fluorophenyl)methylene]hydrazide (**11**)*

Cream-colored solid. MW: 441.926. m.p. 151–153°C. Yield 59%. Rf × 100 value: 41.94 (M_4). FT-IR (ν_{max}, cm^{-1}): 3324, 3232 (indole and hydrazone NH), 1649 (C=O), 1603 (C=N). ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 0.59, 0.66 (3H, tt, $-CH_2-CH_3$ at C_1); 1.26 (3H, t, $-CH_2-CH_3$ at C_8); 2.08–2.17 (2H, m, $-CH_2-CH_3$ at C_1); 2.56–2.68 (2H, m, $-CH_2-CH_3$ at C_8); 2.82–3.68 (4H, m, $-CH_2CONH$ at C_1 and $-CH_2$ at C_4); 3.84–4.02 (2H, m, $-CH_2$ at C_3); 6.87–7.47 (6H, m, Ar–H); 8.25, 8.46 (1H, ss, –N=CH); 10.47 (d, 1H, indole N–H); 11.41, 11.54 (1H, ss, –CO–NH). Anal. calcd. for $C_{24}H_{25}ClFN_3O_2$: C, 65.23; H, 5.70; N, 9.51%. Found: C, 65.50; H, 5.56; N, 9.31%.

*2-(1,8-Diethyl-1,3,4,9-tetrahydropyrano[3,4-b]indole-1-yl)acetic acid[(4-nitrophenyl)methylene]hydrazide (**12**)*

Orange solid. MW: 434.488. m.p. 246°C. Yield 75%. Rf × 100 value: 21.31 (M_2). FT-IR (ν_{max}, cm^{-1}): 3418 (indole and hydrazone NH), 1661 (C=O), 1611 (C=N). ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 0.59, 0.67 (3H, tt, $-CH_2-CH_3$ at C_1); 1.24–1.28 (3H, m, $-CH_2-CH_3$ at C_8); 2.08–2.14 (2H, m, CH_2-CH_3 at C_1); 2.61–2.68 (2H, m, $-CH_2-CH_3$ at C_8); 2.83–3.76 (m, 4H, $-CH_2CONH$ at C_1 and

$-CH_2$ at C_4); 3.78–4.01 (2H, m, $-CH_2$ at C_3); 6.87–8.30 (7H, m, Ar–H); 8.08, 8.34 (1H, ss, N=CH); 10.51 (1H, d, indole N–H); 11.46, 11.57 (1H, ss, CO–NH). Anal. calcd. for $C_{24}H_{26}N_4O_4$: C, 66.34; H, 6.03; N, 12.89%. Found: C, 66.10; H, 5.19; N, 12.83%.

*2-(1,8-Diethyl-1,3,4,9-tetrahydropyrano[3,4-b]indole-1-yl)acetic acid[(pyridine-4-yl)methylene]hydrazide (**13**)*

Yellow solid. MW: 408.493. m.p. 112–113°C. Yield 88%. Rf × 100 value: 15.87 (M_3). FT-IR (ν_{max}, cm^{-1}): 3424, 3308 (indole and hydrazone NH), 1653 (C=O), 1630 (C=N). ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 0.57–0.69 (3H, m, $-CH_2-CH_3$ at C_1); 1.24–1.28 (3H, m, $-CH_2-CH_3$ at C_8); 2.07–2.13 (2H, m, $-CH_2-CH_3$ at C_1); 2.61–2.68 (2H, m, $-CH_2-CH_3$ at C_8); 2.83–3.56 (4H, m, $-CH_2CONH$ at C_1 and $-CH_2$ at C_4); 3.69–4.01 (2H, m, $-CH_2$ at C_3); 6.87–8.33 (m, 7H, Ar–H); 7.96, 8.23 (1H, ss, N=CH); 10.50 (1H, d, indole N–H); 11.42, 11.58 (1H, ss, –CO–NH). Anal. calcd. for $C_{23}H_{26}N_4O_4{\cdot}H_2O$: C, 67.63; H, 6.91; N, 13.72%. Found: C, 67.34; H, 6.60; N, 12.87%.

*2-(1,8-Diethyl-1,3,4,9-tetrahydropyrano[3,4-b]indole-1-yl)acetic acid(phenyl methylene)hydrazide (**14**)*

Light cream-colored solid. MW: 398.498. m.p. 127–129°C. Yield 68%. Rf × 100 value: 34.43 (M_2). FT-IR (ν_{max}, cm^{-1}): 3304 (indole and hydrazone NH), 1639 (C=O), 1605 (C=N). ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 0.59, 0.67 (3H, tt, $-CH_2-CH_3$ at C_1); 1.24–1.29 (3H, m, $-CH_2-CH_3$ at C_8); 2.08–2.16 (2H, m, $-CH_2-CH_3$ at C_1); 2.60–2.68 (2H, m, $-CH_2-CH_3$ at C_8); 2.80–2.99 (4H, m, $-CH_2CONH$ at C_1 and $-CH_2$ at C_4); 3.71–4.01 (2H, m, $-CH_2$ at C_3); 6.88–7.69 (8H, m, Ar–H); 8.00, 8.22 (1H, ss, –N=CH); 10.51 (1H, d, indole N–H); 11.16, 11.34 (1H, ss, CO–NH). Anal. calcd. for $C_{24}H_{27}N_3O_2{\cdot}1/2\ H_2O$: C, 72.33; H, 7.08; N, 10.54%. Found: C, 72.22; H, 6.60; N, 10.32%.

*2-(1,8-Diethyl-1,3,4,9-tetrahydropyrano[3,4-b]indole-1-yl)acetic acid[(3-fluorophenyl)methylene]hydrazide (**15**)*

Light yellow solid. MW: 407.480. m.p. 156–157°C. Yield 86%. Rf × 100 value: 36 (M_2). FT-IR (ν_{max}, cm^{-1}): 3279 (indole and hydrazone NH), 1667 (C=O), 1618 (C=N). ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 0.59, 0.66 (3H, tt, $-CH_2-CH_3$ at C_1); 1.24–1.28 (3H, m, $-CH_2-CH_3$ at C_8); 2.07–2.13 (2H, m, $-CH_2-CH_3$ at C_1); 2.60–2.69 (2H, m, $-CH_2-CH_3$ at C_8); 2.81–3.70 (4H, m, $-CH_2CONH$ at C_1 and $-CH_2$ at C_4); 3.79–4.01 (2H, m, $-CH_2$ at C_3); 6.87–7.51 (7H, m, Ar–H); 7.98, 8.23 (1H, ss, –N=CH); 10.51 (1H, s, indole N–H); 11.28, 11.43 (1H, ss, –CO–NH). Anal. calcd. for $C_{24}H_{26}FN_3O_2$ (407.480): C, 70.74; H, 6.43; N, 10.31%. Found: C, 70.15; H, 6.41; N, 9.92%.

*General procedure for the synthesis of 3-(2-(1,8-diethyl-1,3,4,9-tetrahydropyrano[3,4-b]indole-1-yl)acetylhydrazono)-2-alkyl/aryl-4-thiazolidinones **16–26***

A mixture of compounds **3–15** (0.01 mol) and thioglycolic acid (0.2 mol) was refluxed in dry toluene (100 mL) by using a Dean–Stark water separator. Excess toluene was evaporated under vacuum and the flask content was neutralized by the addition of $NaHCO_3$ (5%) until the CO_2 release was completed. The precipitated solid was washed with water, dried and recrystallized from ethanol/water.

*3-(2-(1,8-Diethyl-1,3,4,9-tetrahydropyrano[3,4-b]indole-1-yl)acetylhydrazono-2-(5-nitro-2-furyl)-4-thiazolidinone (**16**)*

Dark yellow solid. MW: 498.551. m.p. 101–103°C. Yield 29%. Rf × 100 value: 71.43 (M_8). FT-IR (ν_{max}, cm^{-1}): 3325 (indole and amide NH), 1722 (thiazolidinone, C=O), 1668 (amide, C=O). ^{1}H NMR (300 MHz, DMSO-d_6): δ (ppm) 0.54–1.24 (m, 6H, $-CH_2-CH_3$ at C_1 and C_8); 1.89–3.00 (m, 10H, $-CH_2-CH_3$ at C_1 and C_8; $-CH_2-CO-NH$ at C_1; $-CH_2$ at C_3 and C_4); 3.76, 3.89 (2d and each 1H, $-S-CH_2-$, J = 15.6 and 15.9 Hz); 5.15–5.92 (m, 1H, N–CH–S); 6.83–7.67 (m, 5H, Ar–H); 10.13–10.40 (m, 2H, indole NH and –CO–NH–N–). HR-MS (EI$^+$) Calcd./Found (m/z): 498.1573/498.1610 (M^+) ($C_{24}H_{26}N_4O_6S$); 469.1181/469.1192 ($C_{22}H_{21}N_4O_6S$); 467.1930/467.1423; 286.1681/286.1608; 270.1488/270.1434; 242.1539/242.1531; 242.1171/242.1171; 240.1019/240.1048 ($C_{15}H_{14}NO_2$); 229.0157/229.1405; 229.1466/229.1433; 228.1382/228.1382 ($C_{15}H_{18}NO$); 227.1310/227.1322; 212.1069/212.1246.

*3-(2-(1,8-Diethyl-1,3,4,9-tetrahydropyrano[3,4-b]indole-1-yl)-acetylhydrazono)-2-(2-hydroxyphenyl)-4-thiazolidinone (**17**)*

Orange solid. MW: 479.591. m.p. 105–107°C. Yield 65%. Rf × 100 value: 8 (M_5). FT-IR (ν_{max}, cm^{-1}): 3254 (indole and amide NH, OH), 1717 (thiazolidinone, C=O), 1653 (amide, C=O). ^{1}H NMR (500 MHz, DMSO-d_6): δ (ppm) 0.49–1.25 (m, 10H, $-CH_2-CH_3$ at C_1 and C_8); 2.03–3.13 (m, 6H, $-CH_2-CO-NH$ at C_1, $-CH_2$ at C_3 and C_4); 3.66, 4.08 (2d and each 1H, $-S-CH_2-$, J = 16.0 and 16.5 Hz); 5.59–6.06 (m, 1H, –N–CH–S); 6.83–7.39 (m, 7H, Ar–H); 9.89–10.39 (m, 3H, indole NH, –CO–NH–N and –OH). ^{13}C NMR (100 MHz) (DMSO-d_6/TMS): δ (ppm) 8.34 (C-12); 14.57 (C-10); 15.01 (C-9); 22.41 (C-4); 23.76 (C-11); 31.25 (thiazolidinone $S-CH_2-$); 57.37 (C-13); 60.88 (C-3); 65.92 (thiazolidinone –S–CH–); 76.72 (C-1); 108.31 (C-4a); 116.57 (C-1′); 118.72 (C-6); 119.92 (C-5); 120.86 (C-3′); 127.27 (C-6′); 127.84 (C-7); 130.25 (C-5a); 130.57 (C-5′); 135.73 (C-4′); 137.53 (C-8); 139.27 (C-1a); 142.82 (C-8a); 168.50 (C-2′); 171.14 (CO–NH); 172.00 (thiazolidinone C=O). HR-MS (EI$^+$) Calcd./Found (m/z): 479.1873/479.1909 (M^+) ($C_{26}H_{29}N_3O_4S$); 405.2052/405.2036; 270.1488/270.1448; 242.1539/242.1153; 242.1175/242.1153; 240.1019/240.1068 ($C_{15}H_{14}NO_2$); 229.1466/229.1404; 228.1382/228.1366 ($C_{15}H_{18}NO$); 227.1310/227.1304; 212.1069/212.1248; 210.0463/210.1280.

*3-(2-(1,8-Diethyl-1,3,4,9-tetrahydropyrano[3,4-b]indole-1-yl)acetylhydrazono)-2-(4-trifluoromethylphenyl)-4-thiazolidinone (**18**)*

Yellow solid. MW: 531.590. m.p. 99–101°C. Yield 51%. Rf × 100 value: 40.62 (M_5). FT-IR (ν_{max}, cm^{-1}): 3290 (indole and amide NH), 1713 (thiazolidinone, C=O), 1674 (amide, C=O). ^{1}H NMR (300 MHz, DMSO-d_6): δ (ppm) 0.50 (t, 3H, $-CH_2-CH_3$ at C_1); 1.04–2.76 (m, 13H, $-CH_2-CH_3$ at C_8; $-CH_2-CH_3$ at C_1 and C_8; $-CH_2-CO-NH$ at C_1; $-CH_2$ at C_3 and C_4); 3.71, 3.93 (2d and each 1H, $-S-CH_2-$, J = 15.9 Hz); 5.13–5.88 (m, 1H, N–CH–S); 6.84–7.75 (m, 7H, Ar–H); 9.96, 10.06 (ss, 1H, indole NH); 10.31, 10.37 (ss, 1H –CO–NH–N). ^{13}C NMR (75 MHz) (DMSO-d_6/TMS): δ (ppm) 8.11 (C-12); 14.90 (C-10); 22.23 (C-9); 24.16 (C-4); 39.70 (C-11); 31.32 (thiazolidinone $S-CH_2-$); 42.49 (C-13); 60.51 (C-3); 61.44 (thiazolidinone –S–CH–); 75.69 (C-1); 107.71 (C-4a); 115.84 (C-6); 119.19 (C-5); 120.13 (C-7); 124.46 ($-CF_3$); 125.90 (C-8); 126.42 (C-3′ and C-5′); 126.84 (C-5a); 128.92 (C-2′ and C-6′); 129.95 (C-4′); 134.93 (C-1a); 136.51 (C-8a); 143.84 (C-1′); 168.24 (CO–NH); 169.34 (thiazolidinone C=O). HR-MS (EI$^+$) Calcd./Found (m/z): 531.1803/531.1799 (M^+) ($C_{27}H_{28}F_3N_3O_3S$); 512.1814/512.1729; 502.1406/502.1413 ($C_{25}H_{23}F_3N_3O_3S$); 485.1926/485.1299; 270.1488/270.1409; 262.0388/262.9926; 242.1539/242.1199; 242.1175/242.1171; 240.1019/240.1063; 229.1466/229.1443; 228.1382/228.1412 ($C_{15}H_{18}NO$); 227.1310/227.1313; 212.1069/212.1061.

*3-(2-(1,8-Diethyl-1,3,4,9-tetrahydropyrano[3,4-b]indole-1-yl)acetylhydrazono)-2-(2-fluorophenyl)-4-thiazolidinone (**19**)*

Orange solid. MW: 481.582. m.p. 106–108°C. Yield 98%. Rf × 100 value: 31.37 (M_2). FT-IR (ν_{max}, cm^{-1}): 3265 (indole and amide NH), 1720 (thiazolidinone, C=O), 1674 (amide, C=O). ^{1}H NMR (300 MHz, DMSO-d_6): δ (ppm) 0.45–1.22 (m, 6H, $-CH_2-CH_3$ at C_1 and at C_8); 1.84–3.11 (m, 10H, $-CH_2-CH_3$ at C_1 and at C_8; $-CH_2-CO-NH$ at C_1, $-CH_2$ at C_3 and C_4); 3.82, 4.10 (2d and each 1H, $-S-CH_2-$, J = 15.9 Hz,); 5.14–6.02 (m, 1H, N–CH–S); 6.86–7.52 (m, 7H, Ar–H); 10.07–10.50 (m, 2H, indole NH and $-CO-NH-N$). HR-MS (EI^+) Calcd./Found (m/z): 481.1835/481.1849 (M^+) ($C_{26}H_{28}FN_3O_3S$); 463.1929/463.1688; 450.2187/450.1672; 435.1958/435.9727; 270.1488/270.1564; 242.1539/242.1474; 242.1175/242.1168; 240.1019/240.1076; 229.1466/229.1426; 228.1382/228.1374 ($C_{15}H_{18}NO$); 227.1310/227.1301; 212.1069/212.1067.

*3-(2-(1,8-Diethyl-1,3,4,9-tetrahydropyrano[3,4-b]indole-1-yl)acetylhydrazono)-2-(4-fluorophenyl)-4-thiazolidinone (**20**)*

Orange solid. MW: 481.582. m.p. 133–135°C. Yield 75%. Rf × 100 value: 28.30 (M_5). FT-IR (ν_{max}, cm^{-1}): 3298 (indole and amide NH), 1713 (thiazolidinone, C=O), 1674 (amide, C=O). ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 0.51–1.26 (m, 6H, $-CH_2-CH_3$ at C_1 and C_8); 1.6–3.71 (m, 10H, $-CH_2-CH_3$ at C_1 and C_8; $-CH_2-CO-NH$ at C_1; $-CH_2$ at C_3 and C_4); 3.80, 3.84 (2d and each 1H, $-S-CH_2-$, J = 16.02 Hz); 5.71, 5.81 (ss, 1H, N–CH–S); 6.86–7.50 (m, 7H, Ar–H); 9.92, 10.01 (ss, 1H, indole NH); 10.32,10.38 (ss, 1H, $-CO-NH-N$). ^{13}C NMR (100 MHz) (DMSO-d_6/TMS): δ (ppm) 8.19 (C-12); 14.97 (C-10); 22.39 (C-9); 24.30 (C-4); 29.91 (C-11); 31.57 (thiazolidinone $S-CH_2-$); 42.73 (C-13); 60.88 (C-3); 61.90 (thiazolidinone –S–CH–); 76.15 (C-1); 108.29 (C-4a); 116.37 (C-6); 116.54 (C-5); 119.83 (C-3′ ve C-5′); 120.76 (C-7); 127.23 (C-5a); 127.69 (C-2′ ve C-6′); 129.37 (C-8); 131.30 (C-1′); 135.79 (C-1a); 137.27 (C-8a); 165.04 (C-4′); 169.25 (CO–NH); 170.54 (thiazolidinone C=O). HR-MS (EI^+) Calcd./Found (m/z): 481.1835/481.1849 (M^+) ($C_{26}H_{28}FN_3O_3S$); 463.1930/463.1945; 450.2187/450.1684; 452.1439/452.1486 ($C_{24}H_{23}FN_3O_3S$); 435.1958/435.1467; 407.2009/407.2008; 286.1681/286.1189; 270.1488/270.1443; 242.1539/242.1193; 242.1175/242.1193; 240.1019/240.1042 ($C_{15}H_{14}NO_2$); 229.1466/229.1404; 228.1382/228.1383 ($C_{15}H_{18}NO$); 227.1310/227.1297; 212.1069/212.1032; 195.0518/195.0957.

*3-(2-(1,8-Diethyl-1,3,4,9-tetrahydropyrano[3,4-b]indole-1-yl)acetylhydrazono)-2-(2,6-difluorophenyl)-4-thiazolidinone (**21**)*

Yellow solid. MW: 499.573. m.p. 100–102°C. Yield 74%. Rf × 100 value: 33.33 (M_2). FT-IR (ν_{max}, cm^{-1}): 3348 (indole and amide NH), 1715 (thiazolidinone, C=O), 1674 (amide, C=O). ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 0.51–1.40 (m, 6H, $-CH_2-CH_3$ at C_1 and C_8); 1.52–3.81 (m, 10H, $-CH_2-CH_3$ at C_1 and C_8; $-CH_2-CO-NH$ at C_1, $-CH_2$ at C_3 and C_4); 3.85, 4.04 (2d and each 1H, $-S-CH_2-$, J = 15.0 and 16.2 Hz); 6.06, 6.18 (ss, 1H, N–CH–S); 6.69–7.52 (m, 6H, Ar–H); 9.91, 10.0 (ss, 1H, indole NH); 10.30, 10.37 (ss, 1H, $-CO-NH-N$). ^{13}C NMR (100 MHz) (DMSO-d_6/TMS): δ (ppm) 8.25 (C-12); 14.98 (C-10); 22.38 (C-9); 24.31 (C-4); 29.96 (C-11); 31.49 (thiazolidinone $S-CH_2-$); 42.18 (C-13); 60.52 (C-3); 60.91 (thiazolidinone –S–CH–); 76.08 (C-1); 108.35 (C-5′ and C-3′); 113.44 (C-4a); 116.57 (C-1′); 119.92 (C-6); 120.87 (C-5); 127.19 (C-7); 127.26 (C-5a); 127.70 (C-4′); 129.70 (C-8); 132.83 (C-1a); 135.78 (C-8a); 137.81 (C-2′ and C-6′); 163.00 (CO–NH); 169.53 (thiazolidinone C=O). HR-MS (EI^+) Calcd./Found (m/z): 499.1741/499.1763 (M^+) ($C_{26}H_{27}F_2N_3O_3S$); 481.1835/481.1619; 468.2098/468.1543; 463.1930/463.2004; 453.1863/453.1369; 286.1681/286.1460; 270.1488/270.1431; 242.1539/242.1502; 242.1175/242.1139; 240.1019/240.1062 ($C_{15}H_{14}NO_2$); 230.1488/230.1431; 229.1466/229.1398; 228.1382/228.1373 ($C_{15}H_{18}NO$); 227.1310/227.1297; 212.1069/212.1423.

*3-(2-(1,8-Diethyl-1,3,4,9-tetrahydropyrano[3,4-b]indole-1-yl)acetylhydrazono)-2-(4-chlorophenyl)-4-thiazolidinone (**22**)*

Dark orange solid. MW: 498.037. m.p. 91–93°C. Yield 44%. Rf × 100 value: 31.25 (M_5). FT-IR (ν_{max}, cm^{-1}): 3287 (indole and amide NH), 1705 (thiazolidinone, C=O), 1674 (amide, C=O). ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 0.52–3.85 (m, 16H, $-CH_2-CH_3$ at C_1 and C_8; $-CH_2-CO-NH$ at C_1; $-CH_2$ at C_3 and C_4); 3.87, 4.11 (2d and each 1H, $-S-CH_2-$, J = 16.3 and 16.8 Hz); 5.14–5.79 (m, 1H, N–CH–S); 6.62–7.84 (m, 7H, Ar–H); 9.94–10.41 (m, 2H, indole NH and $-CO-NH-N-$). HR-MS (EI^+) Calcd./Found (m/z): 497.1539/497.1487 (M^+) ($C_{26}H_{28}ClN_3O_3S$); 468.1148/468.1201 ($C_{24}H_{23}ClN_3O_3S$); 466.1891/466.1342; 463.1929/463.1892; 451.1662/451.1429; 286.1681/286.1356; 270.1488/270.1456; 242.1539/242.1577; 242.1175/242.1169; 240.1019/240.1075 ($C_{15}H_{14}NO_2$); 229.1466/229.1393; 228.1382/228.1360 ($C_{15}H_{18}NO$); 227.1310/227.1277; 212.1069/212.1037; 194.1681/194.0975.

*3-(2-(1,8-Diethyl-1,3,4,9-tetrahydropyrano[3,4-b]indole-1-yl)acetylhydrazono)-2-(4-methoxyphenyl)-4-thiazolidinone (**23**)*

Yellow solid. MW: 493.617. m.p. 130–133°C. Yield 59%, Rf × 100 value: 44.64 (M_5). FT-IR (ν_{max}, cm^{-1}): 3298 (indole and amide NH), 1705 (thiazolidinone, C=O), 1668 (amide, C=O). ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 0.47–0.63 (m, 3H, $-CH_2-CH_3$ at C_1); 1.04–2.23 (m, 7H, $-CH_2-CH_3$ at C_1 and $-CH_2-CH_3$ at C_8); 2.66–3.70 (m, 6H, $-CH_2-CO-NH$ at C_1, $-CH_2$ at C_3 and C_4) 3.75 (s, 3H, $O-CH_3$); 3.79, 3.85 (2d and each 1H, $-S-CH_2-$, J = 16.7 and 16.0 Hz); 5.66, 5.75 (ss, 1H, –N–CH–S); 6.72–7.37 (m, 7H, Ar–H); 9.90, 9.98 (ss, 1H, indole NH); 10.30, 10.38 (ss, 1H, $-CO-NH-N-$). ^{13}C NMR (75 MHz) (DMSO-d_6/TMS): δ (ppm): 8.30 (C-12); 14.94 (C-10); 22.30 (C-9); 24.21 (C-4); 29.86 (C-11); 30.95 (thiazolidinone $S-CH_2-$); 42.42 (C-13); 55.68 ($-OCH_3$); 60.52 (C-3); 62.03 (thiazolidinone –S–CH–); 75.72 (C-1); 107.60 (C-4a); 114.34 (C-3′ and C-5′); 115.85 (C-6); 119.09 (C-5); 120.13 (C-7); 126.50 (C-8); 127.04 (C-2′ and C-6′); 129.79 (C-5a); 130.16 (C-1′); 134.95 (C-1a); 136.50 (C-8a); 150.24 (C-4′); 168.04 (CO–NH); 169.61 (thiazolidinone C=O). HR-MS (EI^+) Calcd./Found (m/z): 493.2035/493.2029 (M^+) ($C_{27}H_{31}N_3O_4S$); 464.2007/464.2055 ($C_{26}H_{30}N_3O_3S$); 464.1638/464.1715; 463.1929/463.1937 ($C_{26}H_{29}N_3O_3S$); 462.2393/463.1895; 419.2008/419.9784; 286.1681/286.1040; 270.1488/270.1422; 242.1539/242.1639; 242.1175/242.1185; 240.1019/240.1020 ($C_{15}H_{14}NO_2$); 229.1466/229.1398; 228.1382/228.1405 ($C_{15}H_{18}NO$); 227.1310/227.1319; 212.1069/212.1242; 210.0463/210.1187.

*3-(2-(1,8-Diethyl-1,3,4,9-tetrahydropyrano[3,4-b]indole-1-yl)acetylhydrazono)-2-(2-chloro-6-fluorophenyl)-4-thiazolidinone (**24**)*

Yellow solid. MW: 516.027. m.p. 128–130°C. Yield 46%. Rf × 100 value: 32.14 (M_2). FT-IR (ν_{max}, cm^{-1}): 3343 (indole and amide NH), 1709 (thiazolidinone, C=O), 1674 (amide, C=O). ^{1}H NMR

(400 MHz, DMSO-d_6): δ (ppm) 0.51, 0.56 (tt, 3H, $-CH_2-CH_3$ at C_1); 0.84–2.74 (m, 9H, $-CH_2-CH_3$ at C_1, $-CH_2-CH_3$ at C_8, $-CH_2-CO-NH$ at C_1); 2.78–2.96 (m, 4H, $-CH_2$ at C_3 and C_4); 3.76, 3.82 (2d and each 1H, $-S-CH_2-$, $J = 15.7$ and 16.8 Hz); 6.28, 6.41 (ss, 1H, $-N-CH-S$); 6.86–7.49 (m, 6H, Ar–H); 10.13, 10.27 (ss, 1H, indole NH); 10.34, 10.38 (ss, 1H, $-CO-NH-N-$). HR-MS (EI^+) Calcd./Found (*m*/*z*): 515.1445/515.1433 (M^+) ($C_{26}H_{27}ClFN_3O_3S$); 497.1540/497.1388; 486.1049/486.1020 ($C_{26}H_{27}ClFN_3O_3S$); 484.1798/484.1220; 469.1568/469.0902; 463.1930/463.1859; 286.1681/286.1214; 270.1488/270.1465; 246.0030/246.0021; 242.1539/242.1559; 242.1175/242.1184; 240.1019/240.1037 ($C_{15}H_{14}NO_2$); 229.1466/229.1413; 228.1382/228.1384 ($C_{15}H_{18}NO$); 227.1310/227.1323; 212.1069/212.1207.

*3-(2-(1,8-Diethyl-1,3,4,9-tetrahydropyrano[3,4-b]indole-1-yl)acetylhydrazono)-2-(4-nitrophenyl)-4-thiazolidinone (**25**)*

Dark orange solid. MW: 508.589. m.p. 97–100°C. Yield 39%. Rf × 100 value: 16.92 (M_2). FT-IR (ν_{max}, cm^{-1}): 3291 (indole and amide NH), 1709 (thiazolidinone, C=O), 1682 (amide, C=O). ^1H NMR (400 MHz, DMSO-d_6): δ (ppm) 0.83–1.47 (m, 6H, $-CH_2-CH_3$ at C_1 and C_8); 2.64–3.62 (m, 10H, $-CH_2-CH_3$ at C_1 and C_8; $-CH_2-CO-NH$ at C_1; $-CH_2$ at C_3 and C_4); 3.81, 3.87 (2d and each 1H, $-S-CH_2-$, $J = 16.1$ and 17.3 Hz); 5.21–6.12 (m, 1H, $N-CH-S$); 6.83–8.44 (m, 7H, Ar–H); 10.04–10.62 (m, 2H, indole NH and $-CO-NH-N-$). ^{13}C NMR (75 MHz) (DMSO-d_6/TMS): δ (ppm) 14.36 (C-12); 14.95 (C-10); 20.35 (C-9); 23.88 (C-4); 29.49 (C-11); 37.07 (thiazolidinone $S-CH_2-$); 41.20 (C-13); 60.66 (C-3); 61.58 (thiazolidinone $-S-CH-$); 65.67 (C-1); 119.22 (C-4a); 119.95 (C-6); 123.66 (C-5); 124.30 (C-7); 126.77 (C-3′ and C-5′); 127.80 (C-8); 128.33 (C-2′ and C-6′); 128.70 (C-5a); 128.92 (C-1a); 129.35 (C-8a); 147.50 (C-1′); 169.17 (C-4′); 169.69 (CO–NH); 171.02 (thiazolidinone C=O). HR-MS (EI^+) Calcd./Found (*m*/*z*): 508.1780/508.1800 (M^+) ($C_{26}H_{28}N_4O_5S$); 479.1753/479.1775 ($C_{25}H_{27}N_4O_4S$); 477.2132/477.1950; 463.1929/463.1432; 462.1903/462.1538; 435.2026/435.9687; 286.1681/286.1230; 270.1488/270.1459; 242.1539/242.1534; 242.1175/242.1149; 240.1019/240.1205; 239.0364/239.0337; 229.1466/229.0887; 228.1382/228.1390; 227.1310/227.1315; 212.1069/212.1318.

*3-(2-(1,8-Diethyl-1,3,4,9-tetrahydropyrano[3,4-b]indole-1-yl)acetylhydrazono)-2-(4-pyridinyl)-4-thiazolidinone (**26**)*

Orange solid. MW: 464.580. m.p. 101–103°C. Yield 15%. Rf × 100 value: 90 (M_7). FT-IR (ν_{max}, cm^{-1}): 3312 (indole and amide NH), 1713 (thiazolidinone, C=O), 1668 (amide, C=O). ^1H NMR (400 MHz, DMSO-d_6): δ (ppm) 0.51–0.68 (m, 3H, CH_2-CH_3 at C_1); 0.95–2.97 (m, 13H, CH_2-CH_3 at C_1; CH_2-CH_3 at C_8; $-CH_2-CO-NH$ at C_1; $-CH_2$ at C_3 and C_4); 3.94, 4.12 (2d and each 1H, $-S-CH_2-$, $J = 16.0$ and 14.2 Hz); 5.14–5.81 (m, 1H, $N-CH-S$); 6.86–8.70 (m, 7H, Ar–H); 10.04–10.55 (m, 2H, indole NH and $CO-NH-N$). ^{13}C NMR (75 MHz) (DMSO-d_6/TMS): δ (ppm) 8.33 (C-12); 14.90 (C-10); 19.02 (C-9); 22.25 (C-4); 24.18 (C-11); 33.26 (thiazolidinone $S-CH_2-$); 51.07 (C-13); 56.51 (C-3); 61.49 (thiazolidinone $-S-CH-$); 75.74 (C-1); 115.90 (C-4a); 119.21 (C-6); 120.15 (C-5); 121.55 (C-7); 122.87 (C-2′ and C-6′); 123.32 (C-8); 126.44 (C-5a); 127.03 (C-1a); 134.96 (C-8a); 145.89 (C-1′); 149.83 (C-3′ and C-5′); 168.82 (CO–NH); 171.04 (thiazolidinone C=O). HR-MS (EI^+) Calcd./Found (*m*/*z*): 464.1882/464.1859 (M^+) ($C_{25}H_{28}N_4O_3S$); 437.1773/437.2261; 435.1485/435.1473 ($C_{23}H_{23}N_4O_3S$); 433.2234/433.2105; 418.2004/418.9758; 390.2056/390.2208; 286.1681/286.1646; 270.1488/270.1566; 242.1539/242.1370; 242.1175/242.1148; 240.1019/240.1023 ($C_{15}H_{14}NO_2$); 229.1466/229.1414; 228.1382/228.1398 ($C_{15}H_{18}NO$); 227.1310/227.1307; 212.1069/212.1195; 195.0466/195.0536.

Anticancer activity

The primary anticancer assay was performed in accordance with the protocol of the Drug Evaluation Branch, NCI (Bethesda, MD, USA) [44–46, 52]. The human tumor cell lines of the cancer screening panel were grown in RPMI 1640 medium containing 5% fetal bovine serum (FBS) and 2 mM L-glutamine. For a typical screening experiment, 100 μL of cells was inoculated into 96-well microtiter plates at plating densities ranging from 5000 to 40,000 cells/well, depending on the doubling time of the individual cell lines. After cell inoculation, the microtiter plates were incubated at 37°C, 5% CO_2, 95% air and 100% relative humidity for 24 h prior to the addition of the experimental drugs. The cytotoxic and/or growth inhibitory effects of the compounds were tested *in vitro* against the full panel of 60 human tumor cell lines derived from 9 neoplastic diseases, at 10-fold dilution. The percentage of growth was evaluated spectrophotometrically versus controls that were not treated with the test agents. Briefly, the effects of the compounds on the growth parameters of the different cancer cell lines were evaluated relative to controls treated with equivalent amounts of DMSO, and expressed as percent growth rate. The compounds were added at 10^{-5} M concentration for 48 h. Compounds **1**, **9**, **10**, **20**, and **21** chosen as prototypes were evaluated against the full panel of 60 human tumor cell lines at a single dose in the NCI *in vitro* primary anticancer assay.

Cell viability studies (PC-3 cells)

Cell viability effects of etodolac and 2-(1,8-diethyl-1,3,4,9-tetrahydropyrano[3,4-*b*]indole-1-yl)-*N*-[(4-chlorophenyl)methylene]acetohydrazide (compound **9**) were evaluated *in vitro* using the MTT colorimetric method against the prostate cancer cell line PC-3 and the rat fibroblast cell line L-929, at different doses [53, 54], at the Marmara University Faculty of Pharmacy, Department of Pharmaceutical Biotechnology. MTT is cleaved to formazan by the ''succinate-tetrazolium reductase'' system (EC 1.3.99.1), which belongs to the mitochondrial respiratory chain and is active only in viable cells. The PC-3 prostate cancer cell line (ATCC® CRL-1435™) was used for the determination of anticancer activity. This cell was cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Invitrogen) supplemented with 10% FBS (Gibco, Invitrogen), 1% glutamine, and streptomycin-penicillin.

Testing *in vitro* was done by the method of Woerdenbag *et al.* [54] with minor modifications. The MTT metabolic assay was carried out at the seeding density of 1×10^4 cells/well in 96-well flat-bottom cell culture plates with 100 μL of opti-MEM (Invitrogen, USA). Following 24 h of incubation at 37°C, 5% CO_2, the medium was aspirated; the compounds were dissolved in DMSO and diluted with medium before addition to the cell cultures at the concentrations of 5 and 10 μg/mL. The cells were incubated for 48 h at 37°C, 5% CO_2. After the incubation period, 10 μL of the MTT labeling reagent (final concentration 0.5 μg/mL (Cell Proliferation Kit MTT; Roche, Germany)) was added to each well. The samples were incubated for 4–12 h in a humidified atmosphere (*e.g.*, 37°C, 5.0% CO_2) and 100 μL of the solubilization buffer was added into each well. The plate was allowed to stand overnight in the incubator in a humidified atmosphere (*e.g.*,

37°C, 5% CO_2) and the formazan precipitates were then solubilized. The absorbance of the formazan product was measured spectrophotometrically at 550 and 690 nm. Statistical analyses were done using the unpaired Student's *t*-test using Prism 3.0 (GraphPad Software, San Diego, CA, USA).

Apoptosis studies

The apoptosis pathway of etodolac and 2-(1,8-diethyl-1,3,4,9-tetrahydropyrano[3,4-*b*]indole-1-yl)-*N*-[(4-chlorophenyl)methylene]-acetohydrazide **9** was evaluated using the PC-3 prostatic cancer cell line, at the Marmara University Faculty of Pharmacy, Department of Biochemistry. Etodolac and compound **9** were dissolved in DMSO and diluted in culture medium immediately before use. The final concentration of DMSO in all experiments was less than 0.01%, and all treatment conditions were compared with vehicle controls. The cells were incubated with 100 μM etodolac or 100 μM compound **9** for 24, 48, and 72 h at 37°C.

Determination of the mitochondrial membrane potential by spectrofluorimeter

Changes in the mitochondrial membrane potential were measured according to the kit procedure (MitoPT JC-1 911) using the J-aggregate-forming lipophilic cationic fluorochrome JC-1. In healthy cells, the dye accumulates and aggregates in mitochondria, emitting a bright red fluorescence. In apoptotic and necrotic cells with altered mitochondrial membrane potential, the dye remains in the cytoplasm in its monomeric form and the fluorescence is green. Thus, at low membrane potential, JC-1 exists mainly in the green-fluorescent monomeric form.

Briefly, JC-1 was dissolved in 500 μL DMSO for preparing the stock solution and then diluted in phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) on the day of staining. First, cells (1×10^4 cells/well) were incubated in the dark for 30 min at 37°C in the JC-1 working solution. Then, 5 μL JC-1 was added to the tubes and incubated for 20 min in the dark at 37°C. The cells were washed twice with PBS buffer. JC-1-loaded cells were excited at 490 nm, and emission was detected at 510–570 nm (JC-1 aggregates) and 516 nm (JC-1 monomers) using a spectrofluorimeter (GloMax; Promega, USA). Data are presented as relative fluorescence units (RFU).

Monitoring of the mitochondrial membrane potential by fluorescence microscopy

Cells were grown on 12-mm square glass coverslips for the assay, not exceeding a final density of 10^6 cells/mL, and after discarding the culture medium, the JC-1 staining solution was added to the wells. The cells were then incubated at 37°C for 15 min in a CO_2 incubator and washed twice with 1–2 mL assay buffer. A drop of assay buffer was then added to the wells prior to immediate examination in the fluorescence microscope (Olympus, Tokyo, Japan).

Bcl-2 assay

Cells suspended in PBS were permeabilized in digitonine for cytoplasmic staining with a fluorescein isothiocyanate (FITC)-conjugated monoclonal mouse antihuman Bcl-2 antibody (DAKO, Glostrup, Denmark). The samples were incubated with Bcl-2 antibody at room temperature for 30 min. After washing, the cells were resuspended in 1.0 mL PBS containing 0.5% formaldehyde and subjected to spectrofluorimeter analysis (excitation 490 nm, emission 520 nm). The results are presented as RFU/mg protein.

Measurement of caspase-3 activity

Caspase-3 activity was determined using caspase colorimetric assay kits (Sigma, St. Louis, MO, USA). In brief, cells were sonicated in ice-cold lysis buffer. Samples were centrifuged for 10 min at 10,000 × *g* at 4°C. The supernatants were collected, and the protein concentrations were measured by the Bradford method. The caspase-3 assay was based on the spectrophotometric detection of cleavage of the chromophore *p*-nitroanilide (pNA) from Ac-DEVD-pNA. Samples, in duplicate, were incubated for 1 h at 37°C in a buffer mixture containing caspase-3 substrate (Ac-DEVD-pNA) with or without the caspase-3 inhibitor (Ac-DEVD-CHO). pNA (Sigma N2128) was used as the standard. Samples were loaded into each well of a 96-well plate and incubated with Ac-DEVD-pNA at 37°C for 1 h. pNA cleavage was followed spectrophotometrically using an EPOCH microplate reader (Biotec, USA) at 405 nm. The results were expressed as nanomoles of pNA per minute per milligram of protein.

Statistical analysis

Data are presented as mean ± SD. Data were analyzed using repeated measures ANOVA followed by a post-hoc Tukey's test for multiple comparisons. A *p*-value of less than 0.05 was considered statistically significant.

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