Journal of Research in Pharmacy

Research Article

www.jrespharm.com

Synthesis of some novel hydrazide-hydrazones derived from etodolac as potential anti-prostate cancer agents

Hande Cevher KOÇ [1], İrem ATLIHAN [2], Pınar MEGA-TİBER [3], Oya ORUN [3], Ş. Güniz KÜÇÜKGÜZEL [4] *

1 Department of Pharmaceutical Chemistry, Institute of Health Sciences, Marmara University, İstanbul, Turkey.
2 Department of Biophysics, Institute of Health Sciences, Marmara University, İstanbul, Turkey.
3 Department of Biophysics, Faculty of Medicine, Marmara University, İstanbul, Turkey.
4 Vocational School of Health Services, Fenerbahçe University, İstanbul, Turkey
* Corresponding Author. E-mail: guniz.kucukguzel@fbu.edu.tr (Ş.G.K.); Tel. +90-216-910 19 07.

Received: 29 August 2021 / Revised: 27 October 2021 / Accepted: 18 November 2021

ABSTRACT: (*R,S*)-Etodolac [1,8-diethyl-1,3,4,9-tetrahydrapyrano(3,4-*b*)indole-1-acetic acid] is a nonsteroidal anti-inflammatory drug that contains carboxylic acid group with the structure of pyrano[3,4-*b*]indole. In this study, a series of novel (*R,S*)-Etodolac derivatives (**3a-l**) bearing hydrazide-hydrazone moiety were synthesized. The structures of these compounds were characterized by spectral (^{1}H-NMR and FT-IR analyses) methods. All synthesized compounds were screened for anticancer activity against androgen-independent prostate adenocarcinoma (PC-3, DU-145) and androgen-dependent prostate adenocarcinoma (LNCaP) cell lines by using WST-8 colorimetric method. This method was used for cell viability and cytotoxicity analysis. Compound **3b** (SGK-720) [2-(1,8-diethyl-1,3,4,9-tetrahydropyrano[3,4-*b*]indole-1-yl)acetic acid[(2,6-dichlorophenyl)methylene]hydrazides] showed 10.36, 5.24, 15.53 µM anticancer activity against PC3, DU145, LNCaP cancer cell lines, respectively. According to JC-1 mitochondrial membrane potential test and Annexin V/PI staining, **3b** was found to have apoptotic effect on these cancer cells. It is concluded that compound **3b** containing 2,6-dichloro substituents may be one of the candidate molecules to cope with prostate cancer.

KEYWORDS: Etodolac; hydrazide-hydrazone; anticancer activity; apoptosis; WST-8 colorimetric method.

1. INTRODUCTION

Considering the increase in life expectancy and incidence today; prostate cancer has become a public health problem. Prostate cancer is a highly heterogeneous disease with a high incidence, in which both genetic and environmental factors play a role in its etiology. One of the most important features of metastatic cancer cells, which show a high level of genetic and clinical heterogeneity, is that they are resistant to apoptosis and ultimately lead to the development of progressive disease. New scientific research on the mechanisms inducing the apoptotic pathway focuses on the importance of developing synergistic treatment strategies. In the future, it is predicted that the chemotherapeutics will be replaced by molecules that operate physiological mechanisms such as apoptosis and have a low side effect profile.

Epidemiological studies, clinical observations and animal-model studies indicate that NSAIDs have the potential to protect from carcinogenesis. Etodolac [1,8-diethyl-1,3,4,9-tetrahydrapyrano[3,4-*b*]indole-1-acetic acid] has an important antitumor effect on human prostate and bladder cancer cells *in vivo* and *in vitro*. It has been reported to induce apoptosis [1-6].

Hydrazide-hydrazones, which are intermediates in the synthesis of bioactive heterocyclic compounds, have been of wide interest among researchers due to their diverse biological and clinical applications [7]. It is known that the activity of these hydrazone-structure compounds is related to the active pharmacophore group (-CONH-N=C-). However, many studies have been carried out on the *in vivo* and *in vitro* metabolism of hydrazide-hydrazones [8,9] and as a result, the conversion of hydrazones to active metabolites by hydrolysis is a useful method in prodrug synthesis and is less toxic than hydrazides by blocking the -NH_2 group turned out to be [10]. Aldoxorubicin is an albumin binding prodrug derivative of doxorubicin, which binds to an acid-labile maleimide and carries the hydrazone structure. It provides the release of doxorubicin in the acidic

How to cite this article: Koç HC, Atlıhan İ, Mega-Tiber P, Orun O, Küçükgüzel ŞG. Synthesis of some novel hydrazide-hydrazones derived from etodolac as potential anti-prostate cancer agents. J Res Pharm. 2022; 26(1): 1-12.

http://dx.doi.org/10.29228/jrp.97
ISSN: 2630-6344

environment of tumor cells. The reduced toxicity of the conjugate and its ability to tolerate high doses outperformed free doxorubicin.

In recent years, screening studies on anticancer effects on new compounds containing hydrazide-hydrazone have gained importance in order to accelerate the development of new more effective, less toxic chemotherapeutic agents [11-18]. Especially in this period when COX-2 enzyme is an important molecular targeting for anticancer therapies, etodolac (1,8-diethyl-1,3,4,9-tetrahydropyrano[3,4-*b*]indole- 1-yl)acetic acid) has been reported to have anticancer activity against various prostate cancers; Compounds in the structure of hydrazone, which have also been reported in the literature, have been synthesized using etodolac active substance. The structures of new etodolac hydrazide-hydrazone derivatives were elucidated by chromatographic and spectroscopic methods.

2. RESULTS AND DISCUSSION

2.1. Chemistry

The synthesis of twelve novel hydrazide-hydrazone derivatives was carried out by choosing etodolac as a starting compound (Figure 1). Methyl (1,8-diethyl-1,3,4,9-tetrahydropyrano[3,4-*b*]indole-1-yl)acetate (**1**) was prepared by heating etodolac and methanol in the presence of a few drops of concentrated sulfuric acid. 2-(1,8-diethyl-1,3,4,9-tetrahydropyrano[3,4-*b*]indole-1-yl)acetohydrazide (**2**) was obtained by the reaction of compound **1** and hydrazine hydrate in methanol [4]. Compound **2** and substituted benzaldehydes were refluxed in ethanol to synthesize novel 2-(1,8-diethyl-1,3,4,9-tetrahydropyrano[3,4-*b*]indole-1-yl)acetic acid[(substituted phenyl)methylene]hydrazides (**3a-l**).

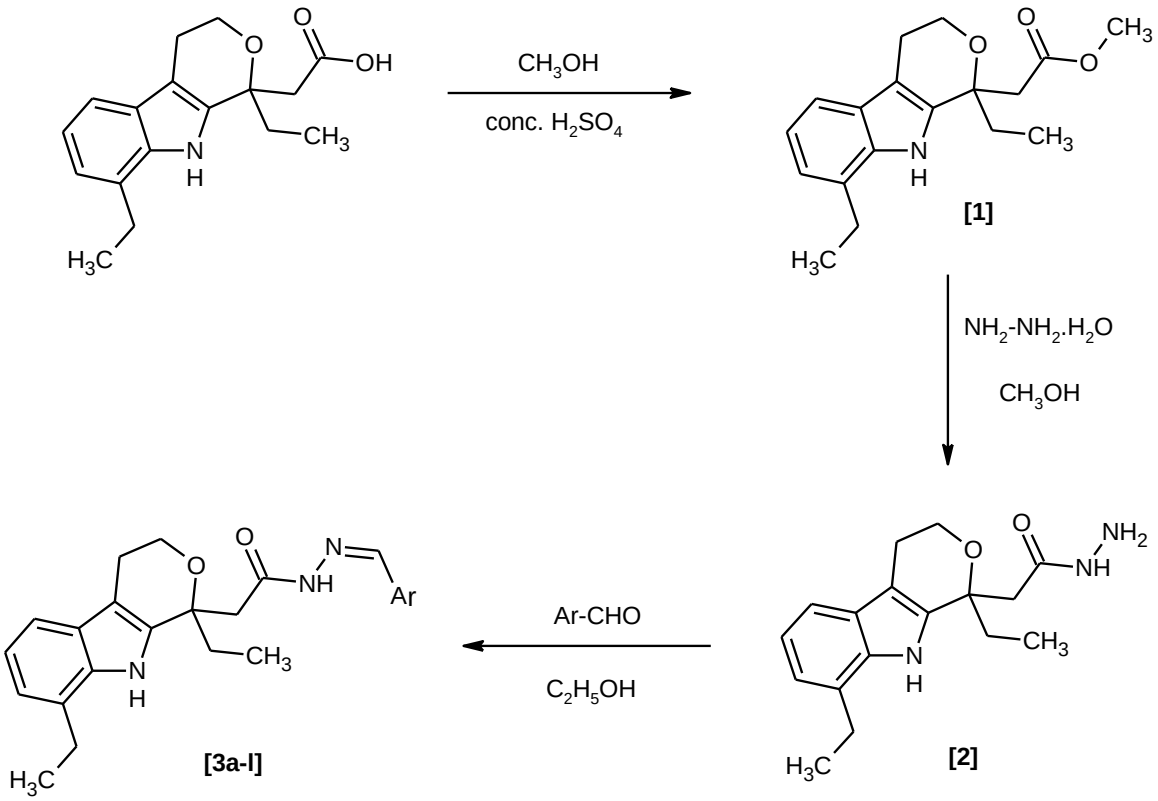

Figure 1. Synthetic route of compounds **3a-l**.

The purity of the synthesized compounds (**3a-l**) was determined by TLC and elemental analysis. The characterization of their structures was proved by FT-IR and ^{1}H-NMR. According to the FT-IR spectra of hydrazide-hydrazones (**3a-l**), C=O and C=N absorption bands appeared at 1673-1641 cm^{-1} and 1617-1566 cm^{-1}, respectively. Also, N-H absorption bands of indole and hydrazone were observed at 3392-3194 cm^{-1}.

The ^{1}H-NMR spectra of compounds **3a**, **3b**, **3d**, **3l** was obtained by using DMSO-d_6 as solvent. Hydrazone N-H and azomethine =CH protons resonated at 9.71-11.59 ppm and 7.64-8.56 ppm, respectively.

The ^{1}H-NMR spectra of other hydrazone compounds except **3a**, **3b**, **3d**, **3l** was studied in $CDCl_3$. Hydrazone N-H protons were detected at 9.12-9.96 ppm. Azomethine =CH protons resonated at 7.67-8.49 ppm. According to the ^{1}H-NMR spectra of compounds which contain methoxy substituent, -OCH_3 protons were observed between 3.80 and 4.02 ppm.

The hydrazones may have both E/Z geometrical isomers around C=N double bonds and cis/trans amide isomers. The hydrazone N-H and azomethine protons of hydrazide derivatives were determined as two singlets instead of a singlet due to isomers [19,20]. Moreover, it reported that hydrazide-hydrazones compounds derived from many compounds in the literature have same situations [4,11,21]. According to obtained information, it is concluded that the synthesized **3a-l** hydrazones exist as E and Z isomers.

2.2. Biological activity

In this study, the anticancer activity of etodolac, etodolac hydrazide (**2**) and hydrazide-hydrazone compounds (**3a-l**) against PC-3, DU-145 and LNCaP prostate cancer cell lines *in vitro* was performed at Department of Biophysics, Faculty of Medicine, Marmara University. The cytotoxic effects of the compounds on these prostate cancer cell lines were evaluated by using WST-8 colorimetric method. The compounds that could be more effective than etodolac and etodolac hydrazide (**2**) were determined according to the IC_{50} values calculated in Table 1.

Table 1. IC_{50} values (μM) of compounds **3a-l**.

Compounds	Lab. code	Ar	PC-3	DU-145	LNCaP
Etodolac			27.21	46.17	19.82
Etodolac Hydrazide (2)			12.37	7.80	41.18
3a	SGK-719	2,4-Dichlorophenyl	28.68	30.38	20.48
3b	SGK-720	2,6-Dichlorophenyl	10.36	5.24	15.53
3c	SGK-721	3,4-Dichlorophenyl	22.31	33.65	25.46
3d	SGK-722	4-Bromophenyl	20.79	11.39	19.81
3e	SGK-723	2-Chloro-3-methoxyphenyl	17.22	21.60	37.91
3f	SGK-724	4-Methoxy-3-nitrophenyl	21.27	30.44	28.94
3g	SGK-725	5-Bromo-2-methoxyphenyl	57.29	45.75	38.70
3h	SGK-726	3-Hydroxy-4-methoxyphenyl	54.82	29.45	67.16
3i	SGK-727	2,5-Dimethoxyphenyl	76.92	40.35	>100
3j	SGK-728	2,6-Dimethoxyphenyl	45.20	43.51	45.89
3k	SGK-729	3,4-Dimethoxyphenyl	25.62	16.51	29.93
3l	SGK-730	2,4,6-Trimethylphenyl	10.20	15.33	34.11

For PC-3 cell line, **3b** and **3l** compounds (IC_{50}=10.36 μM and 10.20 μM) demonstrated higher inhibitory effects than etodolac and etodolac hydrazide (**2**). For DU-145 cell line, the anticancer activity of all hydrazide-hydrazone compounds (**3a-l**) was determined with IC_{50} values of 5.24-45.75 μM. Only **3b** was found to be most effective hydrazide derivative (IC_{50}=5.24 μM), compared to etodolac and etodolac hydrazide (**2**). For LNCaP cell line, **3b** (IC_{50}=15.53 μM) among hydrazone compounds had a better cytotoxic activity than etodolac and etodolac hydrazide (**2**).

Accordingly, it was thought that compound **3b** may have anticancer potential. Then, apoptotic effects of **3b** on PC-3, DU-145 and LNCaP prostate cancer cell lines were evaluated by using JC-1 mitochondrial membrane potential test and Annexin V/PI staining.

Firstly, the effect of **3b** on mitochondrial membrane potential was examined. The same examination was done using etodolac for control. It was seen in Figure 2, Figure 3 and Figure 4 that etodolac has no meaningful effect on mitochondrial membrane potential. In contrast, there was a depolarization tendency in mitochondrial membrane potential with increasing concentrations of compound **3b**.

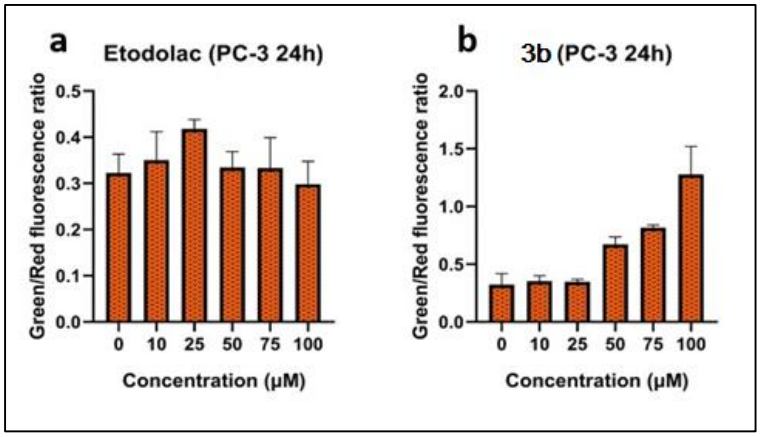

Figure 2. Mitochondrial membrane potential results for **(a)** etodolac and **(b)** compound **3b** in PC-3 cell line.

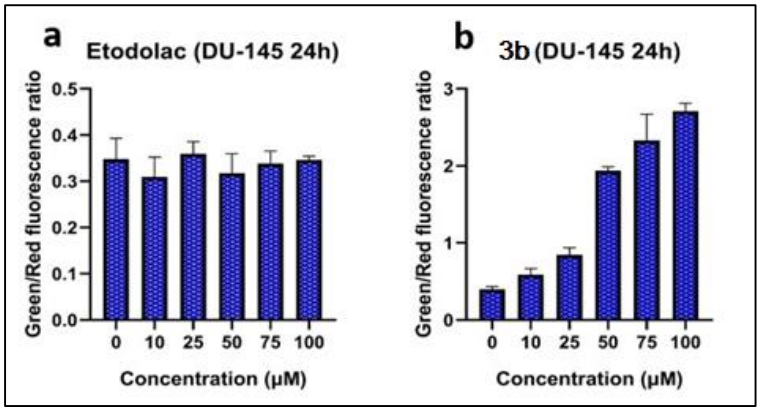

Figure 3. Mitochondrial membrane potential results for **(a)** etodolac and **(b)** compound **3b** in DU-145 cell line.

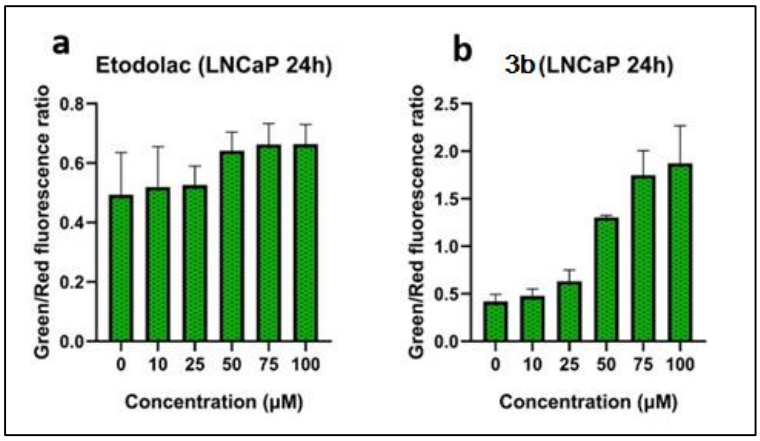

Figure 4. Mitochondrial membrane potential results for **(a)** etodolac and **(b)** compound **3b** in LNCaP cell line.

Secondly, dominant type of cell death on the PC-3, DU-145 and LNCaP cancer cells was determined by the help of Annexin V/PI staining. After treatment with 10 μM etodolac hydrazide (**2**) and 10 μM compound **3b** for 24 hours, the number of cells leading to necrosis and apoptosis increased with comparing to the control group in the PC-3 cell line stained with Annexin V and PI. The number of PC-3 cells undergoing necrosis after incubation with **3b** compound was approximately 35% less than the number of cells undergoing necrosis after incubation with etodolac hydrazide (**2**). In addition, the number of PC-3 cells undergoing apoptosis after incubation with **3b** was 12% higher than with etodolac hydrazide (**2**). When the hydrazide and compound **3b**, which have IC_{50} values close to each other, were evaluated in this respect, it was clear that the compound **3b** causes apoptotic cell death. The effect of etodolac hydrazide (**2**) was mostly associated with necrotic cell death. The PC-3 cells examined under fluorescence microscope were shown in Figure 5.

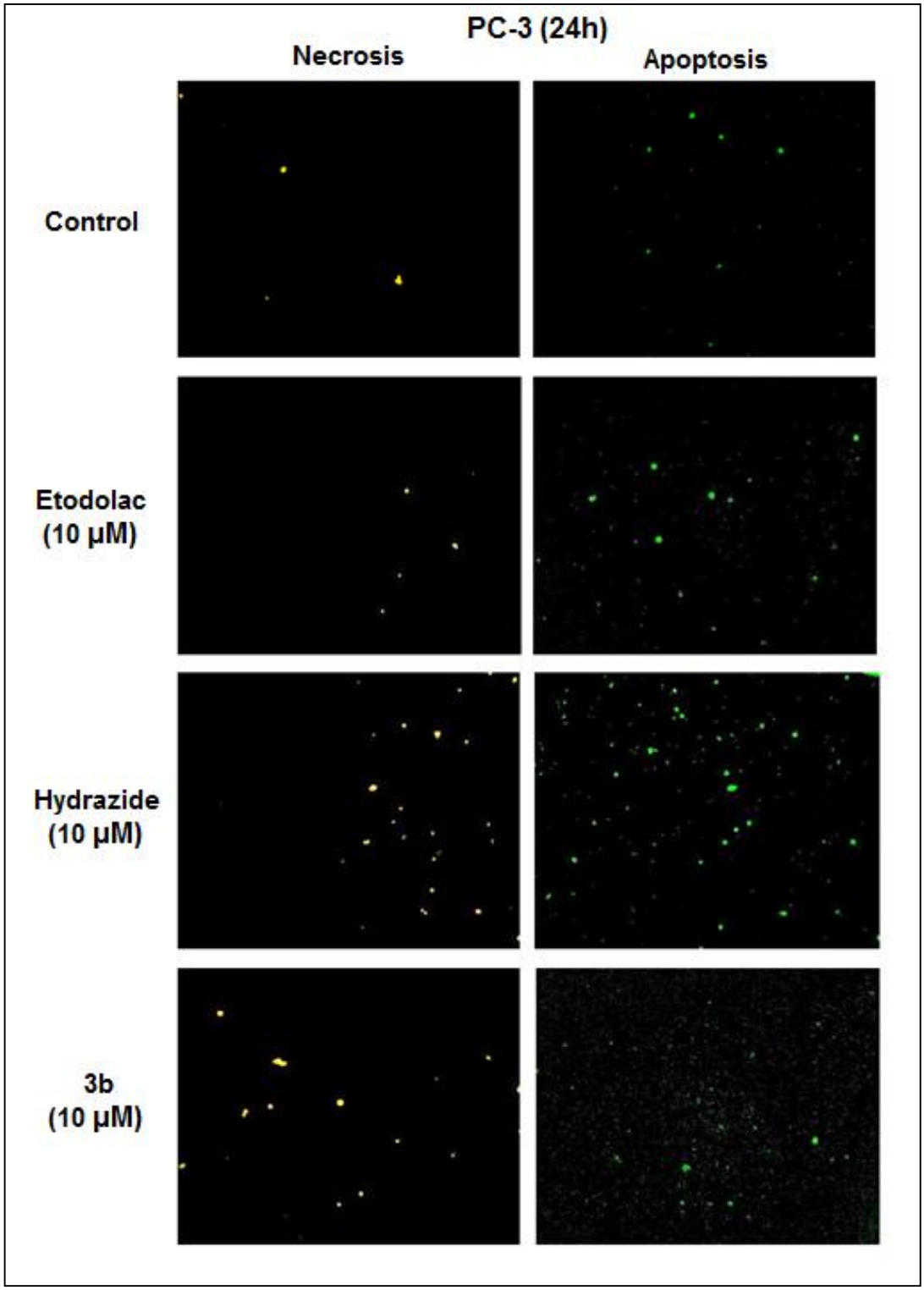

Figure 5. PC-3 cell line undergoing necrosis and apoptosis under fluorescence microscopy.

When the same procedure was performed in the DU-145 cell line, the number of cells undergoing necrosis was approximately 12% higher for compound **3b** than for etodolac hydrazide (**2**). In addition, the number of cells undergoing apoptosis was approximately 21% higher. These results were considered to be compatible with the IC_{50} values. DU-145 cells examined under fluorescence microscope were shown in Figure 6.

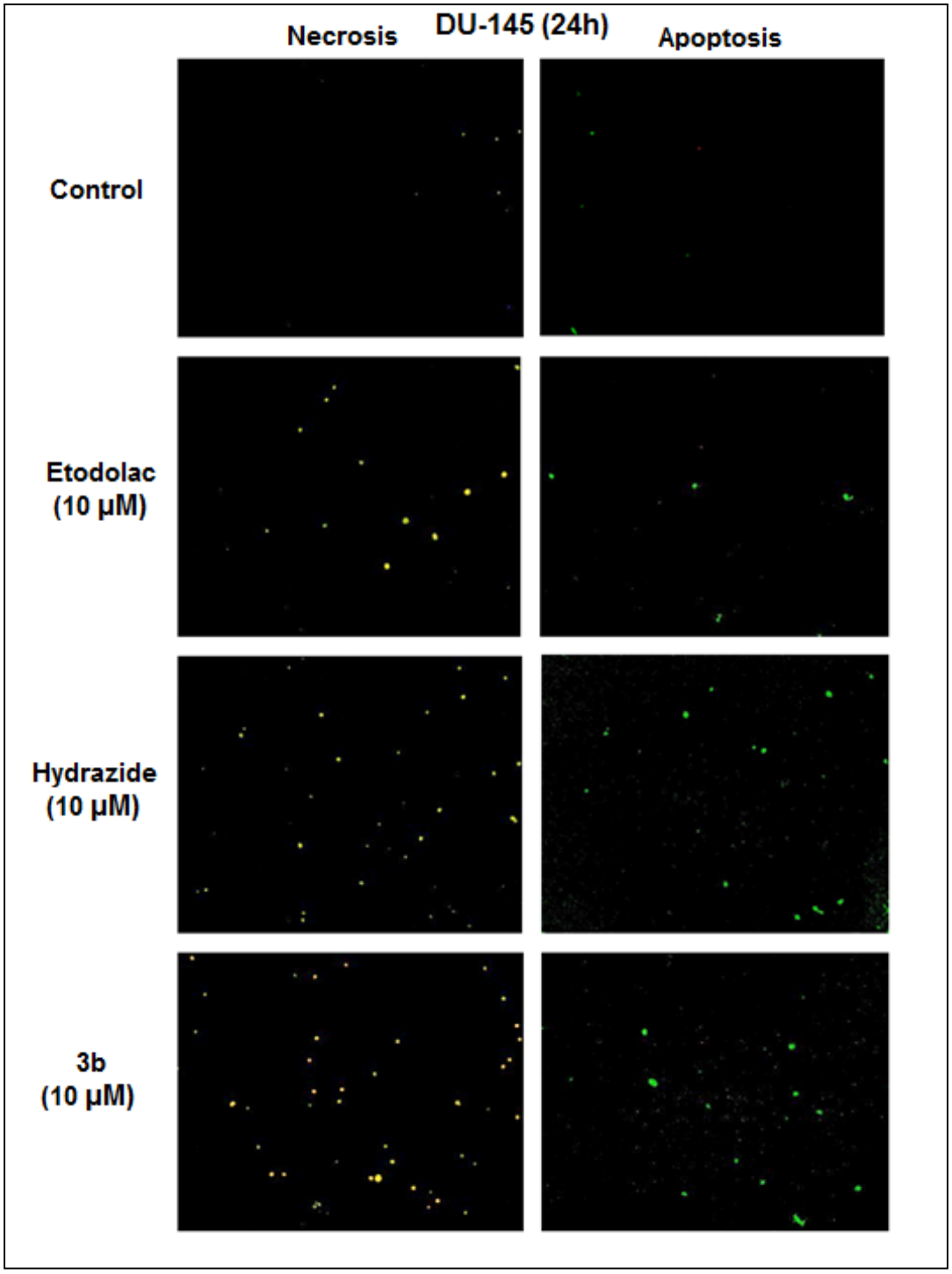

Figure 6. DU-145 cell line undergoing necrosis and apoptosis under fluorescence microscopy.

According to the IC_{50} values determined in the LNCaP cell line, the compounds were prepared and applied at a concentration of 25 µM. Etodolac and compound **3b** gave the most effective results in the LNCaP cell line stained with Annexin V and PI after 24 hours of incubation. Especially in the LNCaP cell line treated with **3b**, the number of cells undergoing apoptosis increased almost nine times compared to the control group. In addition, after incubation with etodolac, the number of LNCaP cells undergoing necrosis was approximately 60% higher compared to compound **3b**; the number of cells undergoing apoptosis was approximately 46% less. According to these results, etodolac caused more necrotic cell death, whereas compound **3b** caused apoptotic cell death. LNCaP cells examined under fluorescence microscope were shown in Figure 7.

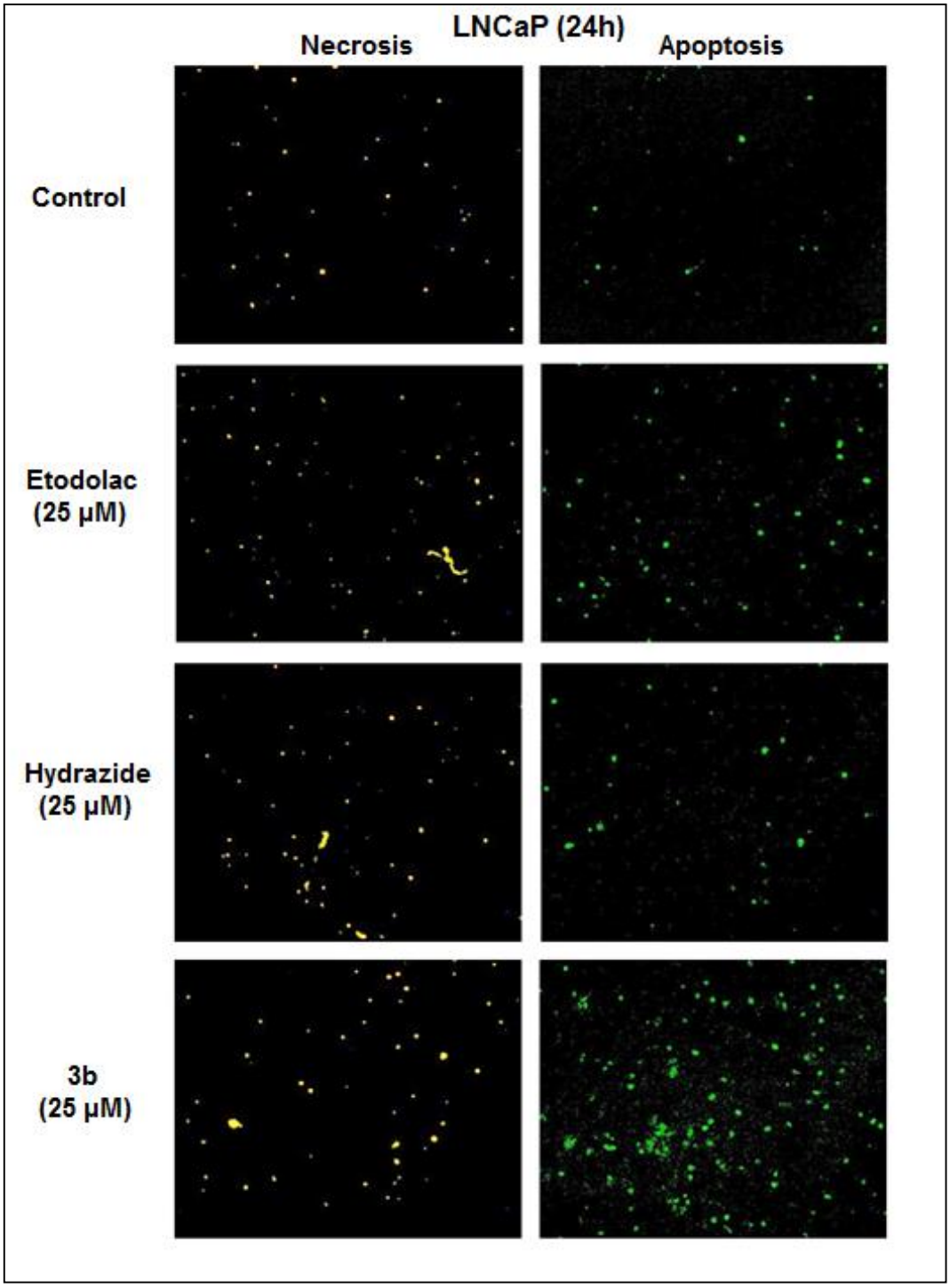

Figure 7. LNCaP cell line undergoing necrosis and apoptosis under fluorescence microscopy.

3. CONCLUSION

In this study, possible anticancer activity of synthesized twelve novel etodolac hydrazide-hydrazones (**3a-l**) was evaluated *in vitro* against three prostate cancer cell lines. According to the results of WST-8 assay, only compound **3b** demonstrated the better cytotoxic effect than etodolac and etodolac hydrazide (**2**) on PC-3, DU-145 and LNCaP cell lines with IC_{50} values of 10.36 µM, 5.24 µM and 15.53 µM, respectively. As a result of JC-1 mitochondrial membrane potential test and Annexin V/PI staining, the apoptotic effect of compound **3b** on these cancer cell lines was proved. Therefore, these results may be a preliminary study and suggest that the compound **3b** may have anticancer effects on prostate cancer.

4. MATERIALS AND METHODS

4.1. Chemistry

All chemicals were purchased from Merck, Sigma-Aldrich, Fluka. Reactions were monitored by thin layer chromatography (TLC) on silica gel plates purchased from Merck. Melting points of the synthesized compounds were determined in Schmelzpunktbestimmer SMP II melting point apparatus and are uncorrected. The purity of the compounds was checked on TLC plates pre-coated with silica gel G using the solvent systems M_1 (petroleum ether/ethyl acetate 60:40 v/v) and M_2 (petroleum ether/ethyl acetate 30:70 v/v). The spots were located under UV light (254 nm) (t: 25°C). FT-IR spectra were recorded on a Perkin Elmer FT-IR Spectrum BX spectrophotometer. ^{1}H-NMR spectra were recorded on Bruker 300 MHz Ultrashield TM spectrometer using DMSO-d_6 or $CDCl_3$ as solvent. Elemental analyses were performed on a LECO CHNS-932. Chemical shifts (δ) are reported in parts per million (ppm). Data are reported as follows: chemical shift, multiplicity (s: singlet, d: doublet, t: triplet, q: quartet, m: multiplet), coupling constants (Hz), and integration.

*4.1.1. Preparation of methyl (1,8-diethyl-1,3,4,9-tetrahydropyrano[3,4-b]indole-1-yl)acetate (**1**, CAS Number: 122188-02-7) and 2-(1,8-diethyl-1,3,4,9-tetrahydropyrano[3,4-b]indole-1-yl)acetohydrazide (**2**, CAS Number: 946848-58-4)*

Etodolac (0.01 mol) and methanol (16 ml) were heated under reflux for 3 h in a few drops of concentrated sulfuric acid. After the reaction mixture was cooled and neutralized by using $NaHCO_3$ (5%), the resulting precipitate was filtered, dried, and recrystallized twice from ethanol. (For compound **1** mp 128-130°C [22,23]). This compound has been previously studied by Çıkla et al. [4].

Compound **1** (0.01 mol) and hydrazine-hydrate (80%, 7 ml) were refluxed in 20 ml methanol for 3 h. After the reaction mixture was cooled, it was diluted with water and allowed to stand overnight. The obtained precipitate was washed with water, dried, and recrystallized twice from petroleum ether. (For compound **2** mp 186-188°C [4]). This compound has been previously studied by Çıkla et al. [4].

*4.1.2. General procedure for the synthesis of 2-(1,8-diethyl-1,3,4,9-tetrahydropyrano[3,4-b]indole-1-yl)acetic acid[(substituted phenyl)methylene]hydrazides (**3a-l**)*

Compound **2** (0.01 mol) and an appropriate substituted benzaldehyde (0.01 mol) were refluxed in 30 ml ethanol for 3 h. The precipitated product was filtered off, dried, and recrystallized twice from ethanol to obtaine novel 2-(1,8-diethyl-1,3,4,9-tetrahydropyrano[3,4-*b*]indole-1-yl)acetic acid[(substituted phenyl)methylene]hydrazides (**3a-l**).

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

Light cream-colored solid. Yield: 44.77%. Mp: 177-178°C. R_f: 0.63 (M_1). FT-IR (ν_{max}, cm^{-1}): 3244, 3194 (indole and hydrazone N-H), 1669 (C=O), 1605 (C=N). ^{1}H-NMR (300 MHz, DMSO-d_6): δ (ppm) 0.59, 0.66 (3H, tt, -CH_2-C<u>H</u>$_3$ at C_1), 1.25 (3H, t, -CH_2-C<u>H</u>$_3$ at C_8), 2.05-2.16 (2H, m, -C<u>H</u>$_2$-CH_3 at C_1), 2.58-2.70 (2H, m, -C<u>H</u>$_2$-CH_3 at C_8), 2.81-3.71 (4H, m, -C<u>H</u>$_2$-CO-NH- at C_1 and -C<u>H</u>$_2$- at C_4), 3.81-4.01 (2H, m, -C<u>H</u>$_2$ at C_3), 6.85-7.95 (6H, m, Ar-H), 8.33, 8.56 (1H, ss, -N=C<u>H</u>), 10.51 (1H, s, indole N-H), 11.54 (1H, d, J = 19.8 Hz, -CO-N<u>H</u>-). Anal. calcd. for $C_{24}H_{25}Cl_2N_3O_2$ (458.380): C: 62.89, H: 5.50, N: 9.17. Found: C: 62.37, H: 5.44, N: 9.13.

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

White solid. Yield: 51.74%. Mp: 166°C. R_f: 0.66 (M_1). FT-IR (ν_{max}, cm^{-1}): 3354, 3316, 3245 (indole and hydrazone N-H), 1651 (C=O), 1583 (C=N). ^{1}H-NMR (300 MHz, DMSO-d_6): δ (ppm) 0.60, 0.67 (3H, tt, -CH_2-C<u>H</u>$_3$ at C_1), 1.23-1.29 (3H, m, -CH_2-C<u>H</u>$_3$ at C_8), 2.05-2.16 (2H, m, -C<u>H</u>$_2$-CH_3 at C_1), 2.59-2.82 (2H, m, -C<u>H</u>$_2$-CH_3 at C_8), 2.85-3.63 (4H, m, -C<u>H</u>$_2$-CO-NH- at C_1 and -C<u>H</u>$_2$- at C_4), 3.78-4.00 (2H, m, -C<u>H</u>$_2$ at C_3), 6.86-7.56 (6H, m, Ar-H), 8.22, 8.42(1H, ss, -N=C<u>H</u>), 10.47 (1H, d, J = 15.3 Hz, indole N-H), 11.43, 11.59 (1H, ss, -CO-N<u>H</u>-). Anal. calcd. for $C_{24}H_{25}Cl_2N_3O_2$ (458.380): C: 62.89, H: 5.50, N: 9.17. Found: C: 62.21, H: 5.37, N: 9.18.

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

White solid. Yield: 49.78%. Mp: 197-201°C. R_f: 0.48 (M_1). FT-IR (ν_{max}, cm^{-1}): 3750-3400 (O-H), 3232 (indole and hydrazone N-H), 1673 (C=O), 1587 (C=N). ^{1}H-NMR (300 MHz, $CDCl_3$): δ (ppm) 0.88-0.98 (3H, m, -CH_2-C<u>H</u>$_3$ at C1), 1.24-1.37 (3H, m, -CH_2-C<u>H</u>$_3$ at C_8), 2.02-2.27 (2H, m, -C<u>H</u>$_2$-CH_3 at C_1), 2.79-3.71 (6H, m, -C<u>H</u>$_2$-CH_3 at C_8, -C<u>H</u>$_2$-CO-NH at C_1 and -C<u>H</u>$_2$- at C_4), 4.07-4.19 (2H, m, -C<u>H</u>$_2$ at C_3), 6.99-7.51, 7.79-7.93 (6H, m, Ar-H), 7.68, 7.74 (1H, ss, -N=C<u>H</u>), 9.40, 9.54 (1H, ss, indole N-H), 9.12, 9.86 (1/2 H, ss, -CO-N<u>H</u>-) Anal. calcd. for $C_{24}H_{25}Cl_2N_3O_2.1/2H_2O$ (467.388): C: 61.67, H: 5.61, N: 8.99. Found: C: 61.93, H: 5.04, N: 9.05.

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

White solid. Yield: 46.15%. Mp: 182ºC. R_f: 0.43 (M_1). FT-IR (ν_{max}, cm^{-1}): 3233, 3213, 3198 (indole and hydrazone N-H), 1667 (C=O), 1612 (C=N). ^{1}H-NMR (300 MHz, DMSO-d_6): δ (ppm) 0.58, 0.66 (3H, tt, -CH_2-C$\underline{H}_3$ at C1), 1.23-1.29 (3H, m, -CH_2-C$\underline{H}_3$ at C_8), 2.05-2.17 (2H, m, -C$\underline{H}_2$-CH_3 at C_1), 2.60-2.73 (2H, m, -C$\underline{H}_2$-CH_3 at C_8), 2.80-3.75 (4H, m, -C$\underline{H}_2$-CO-NH- at C_1 and -C$\underline{H}_2$- at C_4), 3.80-4.04 (2H, m, -C$\underline{H}_2$ at C_3), 6.87-7.63 (7H, m, Ar-H), 7.97, 8.21 (1H, ss, -N=C$\underline{H}$), 10.51 (1H, d, J = 2.4 Hz, indole N-H), 11.24, 11.41 (1H, ss, -CO-N$\underline{H}$-). Anal. calcd. for $C_{24}H_{26}BrN_3O_2$ (468.386): C: 61.54, H: 5.60, N: 8.97. Found: C: 61.68, H: 5.37, N: 9.03.

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

Light cream-colored solid. Yield: 52.34%. Mp: 183ºC. R_f: 0.58 (M_1). FT-IR (ν_{max}, cm^{-1}): 3750-3400 (O-H), 3240, 3220 (indole and hydrazone N-H), 1669 (C=O), 1566 (C=N). ^{1}H-NMR (300 MHz, $CDCl_3$): δ (ppm) 0.87-0.96 (3H, m, -CH_2-C$\underline{H}_3$ at C_1), 1.26-1.39 (3H, m, -CH_2-C$\underline{H}_3$ at C_8), 2.07-2.31 (2H, m, -C$\underline{H}_2$-CH_3 at C_1), 2.80-3.72 (6H, m, -C$\underline{H}_2$-CH_3 at C_8, -C$\underline{H}_2$-CO-NH at C_1 and -C$\underline{H}_2$- at C_4), 3.94 (3H, d, J = 3 Hz, -OC$\underline{H}_3$), 4.07-4.16 (2H, m, -C$\underline{H}_2$ at C_3), 6.94-7.75 (6H, m, Ar-H), 8.28, 8.49 (1H, ss, -N=C$\underline{H}$), 9.11, 9.18 (1H, ss, indole N-H), 9.63, 9.81 (1H, ss, -CO-N$\underline{H}$-). Anal. calcd. for $C_{25}H_{28}ClN_3O_3.1/2H_2O$ (462.969): C: 64.86, H: 6.31, N: 9.08. Found: C: 65.23, H: 5.79, N: 9.24.

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

Dark yellow solid. Yield: 36.85%. Mp: 162-165ºC. R_f: 0.63 (M_2). FT-IR (ν_{max}, cm^{-1}): 3750-3400 (O-H), 3342, 3289 (indole and hydrazone N-H), 1649 (C=O), 1617 (C=N). ^{1}H-NMR (300 MHz, $CDCl_3$): δ (ppm) 0.89, 0.95 (3H, tt, -CH_2-C$\underline{H}_3$ at C_1), 1.24-1.36 (3H, m, -CH_2-C$\underline{H}_3$ at C_8), 2.02-2.30 (2H, m, -C$\underline{H}_2$-CH_3 at C_1), 2.76-3.70 (6H, m, -C$\underline{H}_2$-CH_3 at C_8, -C$\underline{H}_2$-CO-NH at C_1 and -C$\underline{H}_2$- at C_4), 3.98, 4.02 (3H, ss, -OC$\underline{H}_3$), 4.09-4.18 (2H, m, -C$\underline{H}_2$ at C_3), 6.98-7.97 (6H, m, Ar-H), 8.05 (1H, t, -N=C$\underline{H}$), 9.50, 9.55 (1H, ss, indole N-H), 9.21, 9.96 (1H, ss, -CO-N$\underline{H}$-). Anal. calcd. for $C_{25}H_{28}N_4O_5.1/2H_2O$ (473.522): C: 63.41, H: 6.17, N: 11.83. Found: C: 63.19, H: 6.38, N: 11.63.

*2-(1,8-Diethyl-1,3,4,9-tetrahydropyrano[3,4-b]indole-1-yl)acetic acid[(5-bromo-2-methoxyphenyl)methylene]hydrazides (**3g**)*

White solid. Yield: 43.17%. Mp: 164-165ºC. R_f: 0.81 (M_2). FT-IR (ν_{max}, cm^{-1}): 3750-3400 (O-H), 3243, 3202 (indole and hydrazone N-H), 1645 (C=O), 1595 (C=N). ^{1}H-NMR (300 MHz, $CDCl_3$): δ (ppm) 0.92 (3H, q, -CH_2-C$\underline{H}_3$ at C_1), 1.28-1.37 (3H, m, -CH_2-C$\underline{H}_3$ at C_8), 2.05-2.32 (2H, m, -C$\underline{H}_2$-CH_3 at C_1), 2.81-3.72 (6H, m, -C$\underline{H}_2$-CH_3 at C_8, -C$\underline{H}_2$-CO-NH at C_1 and -C$\underline{H}_2$- at C_4), 3.82, 3.86 (3H, ss, -OC$\underline{H}_3$), 4.10-4.18 (2H, m, -C$\underline{H}_2$ at C_3), 6.78-8.01, 8.18-8.19 (6H, m, Ar-H), 8.17, 8.34 (1H, ss, -N=C$\underline{H}$), 9.24, 9.36(1H, ss, indole N-H), 9.66 (1H, d, J = 4.5 Hz, -CO-N$\underline{H}$-). Anal. calcd. for $C_{25}H_{28}BrN_3O_3.1/2H_2O$ (507.420): C: 59.18, H: 5.76, N: 8.28. Found: C: 59.24, H: 5.19, N: 8.35.

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

Dark cream-colored solid. Yield: 24.59%. Mp: 110ºC. R_f: 0.71 (M_2). FT-IR (ν_{max}, cm^{-1}): 3279 (indole and hydrazone N-H, O-H), 1652 (C=O), 1610 (C=N). ^{1}H-NMR (300 MHz, $CDCl_3$): δ (ppm) 0.90 (3H, q, -CH_2-C$\underline{H}_3$ at C_1), 1.25-1.38 (3H, m, -CH_2-C$\underline{H}_3$ at C_8), 2.05-2.30 (2H, m, -C$\underline{H}_2$-CH_3 at C_1), 2.81-3.73 (6H, m, -C$\underline{H}_2$-CH_3 at C_8, -C$\underline{H}_2$-CO-NH at C_1 and -C$\underline{H}_2$- at C_4), 3.92 (3H, d, J = 10.5 Hz, -OC$\underline{H}_3$), 4.08-4.16 (2H, m, -C$\underline{H}_2$ at C_3), 5.87 (1H, s, -OH), 6.81-7.41 (6H, m, Ar-H), 7.67, 7.88 (1H, ss, -N=C$\underline{H}$), 9.18, 9.38 (1H, ss, indole N-H), 9.64, 9.70 (1H, ss, -CO-N$\underline{H}$-). Anal. calcd. for $C_{25}H_{29}N_3O_4.H_2O$ (453.531): C: 66.21, H: 6.89, N: 9.27. Found: C: 66.46, H: 7.22, N: 9.09.

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

White solid. Yield: 26.67%. Mp: 170-171ºC. R_f: 0.79 (M_2). FT-IR (ν_{max}, cm^{-1}): 3750-3400 (O-H), 3244, 3222, 3202 (indole and hydrazone N-H), 1643 (C=O), 1601 (C=N). ^{1}H-NMR (300 MHz, $CDCl_3$): δ (ppm) 0.91 (3H, q, -CH_2-C$\underline{H}_3$ at C_1), 1.27-1.38 (3H, m, -CH_2-C$\underline{H}_3$ at C_8), 2.06-2.27 (2H, m, -C$\underline{H}_2$-CH_3 at C_1), 2.78-3.79 (6H, m, -C$\underline{H}_2$-CH_3 at C_8, -C$\underline{H}_2$-CO-NH at C_1 and -C$\underline{H}_2$- at C_4), 3.80, 3.84 (6H, dd, J = 3.3 Hz, J = 4.2 Hz, -OC$\underline{H}_3$), 4.05-4.17 (2H, m, -C$\underline{H}_2$ at C_3), 6.84-7.57 (6H, m, Ar-H), 8.21, 8.42 (1H, ss, -N=C$\underline{H}$), 9.10, 9.27 (1H, ss, indole N-H), 9.57, 9.72 (1H, ss, -CO-N$\underline{H}$-). Anal. calcd. for $C_{26}H_{31}N_3O_4.1/2H_2O$ (458.550): C: 68.10, H: 7.03, N: 9.16. Found: C: 68.25, H: 6.28, N: 9.19.

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

Cream-colored solid. Yield: 55.99%. Mp: 194-195°C. R_f: 0.69 (M_2). FT-IR (ν_{max}, cm^{-1}): 3750-3400 (O-H), 3392, 3323, 3230 (indole and hydrazone N-H), 1651 (C=O), 1606 (C=N). ^{1}H-NMR (300 MHz, $CDCl_3$): δ (ppm) 0.89-0.94 (3H, m, -CH_2-C<u>H</u>$_3$ at C_1), 1.25-1.40 (3H, m, -CH_2-C<u>H</u>$_3$ at C_8), 2.06-2.32 (2H, m, -C<u>H</u>$_2$-CH_3 at C_1), 2.75-3.77 (6H, m, -C<u>H</u>$_2$-CH_3 at C_8, -C<u>H</u>$_2$-CO-NH at C_1 and -C<u>H</u>$_2$- at C_4), 3.85, 3.89 (6H, ss, -OC<u>H</u>$_3$), 4.10-4.14 (2H, m, -C<u>H</u>$_2$ at C_3), 6.57-7.42 (6H, m, Ar-H), 8.17, 8.44 (1H, ss, -N=C<u>H</u>), 9.06, 9.25 (1H, ss, indole N-H), 9.55, 9.64 (1H, ss, -CO-N<u>H</u>-). Anal. calcd. for $C_{26}H_{31}N_3O_4.1/2H_2O$ (458.550): C: 68.10, H: 7.03, N: 9.16. Found: C: 68.29, H: 6.20, N: 9.21.

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

Light cream-colored solid. Yield: 45.74%. Mp: 140-142°C. R_f: 0.60 (M_2). FT-IR (ν_{max}, cm^{-1}): 3750-3400 (O-H), 3286, 3252, 3208 (indole and hydrazone N-H), 1641 (C=O), 1600 (C=N). ^{1}H-NMR (300 MHz, $CDCl_3$): δ (ppm) 0.91 (3H, q, -CH_2-C<u>H</u>$_3$ at C_1), 1.26, 1.35 (3H, tt, -CH_2-C<u>H</u>$_3$ at C_8), 2.07-2.31 (2H, m, -C<u>H</u>$_2$-CH_3 at C_1), 2.78-3.75 (6H, m, -C<u>H</u>$_2$-CH_3 at C_8, -C<u>H</u>$_2$-CO-NH at C_1 and -C<u>H</u>$_2$- at C_4), 3.89-3.96 (6H, m, -OC<u>H</u>$_3$), 4.08-4.16 (2H, m, -C<u>H</u>$_2$ at C_3), 6.83-7.41 (6H, m, Ar-H), 7.73, 7.94 (1H, ss, -N=C<u>H</u>), 9.19, 9.34 (1H, ss, indole N-H), 9.70 (1H, d, J = 11.7 Hz, -CO-N<u>H</u>-). Anal. calcd. for $C_{26}H_{31}N_3O_4.H_2O$ (467.558): C: 66.79, H: 7.11, N: 8.99. Found: C: 67.03, H: 6.62, N: 8.81.

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

White solid. Yield: 43.83%. Mp: 173°C. R_f: 0.35, 0.63 (M_1). FT-IR (ν_{max}, cm^{-1}): 3271, 3199 (indole and hydrazone N-H), 1663 (C=O), 1610 (C=N). ^{1}H-NMR (300 MHz, DMSO-d_6): δ (ppm) 0.59-0.70 (3H, m, -CH_2-C<u>H</u>$_3$ at C_1), 1.21-1.29 (3H, m, -CH_2-C<u>H</u>$_3$ at C_8), 2.08-2.37 (11H, m, -C<u>H</u>$_2$-CH_3 at C_1 and phenyl -C<u>H</u>$_3$), 2.58-2.72 (2H, m, -C<u>H</u>$_2$-CH_3 at C_8), 2.77-3.48 (4H, m, -C<u>H</u>$_2$-CO-NH- at C_1 and -C<u>H</u>$_2$- at C_4), 3.86-4.02 (2H, m, -C<u>H</u>$_2$ at C_3), 6.86-7.26 (5H, m, Ar-H), 7.64, 8.34, 8.46 (1H, sss, -N=C<u>H</u>), 10.43-10.53 (1H, m, indole N-H), 9.71, 11.03, 11.16 (1H, sss, -CO-N<u>H</u>-). Anal. calcd. for $C_{27}H_{33}N_3O_2$ (431.569): C: 75.14, H: 7.71, N: 9.74. Found: C: 74.68, H: 7.27, N: 9.88.

4.2. Anticancer activity

4.2.1. Cell viability and cytotoxicity studies

The effects of the compounds on cell viability and cytotoxicity in three different prostate cancer cell lines were investigated *in vitro*. PC-3, DU-145 and LNCaP prostate cancer cells were incubated at 37 °C, 5% CO_2 with RPMI-1640 medium. The cells were studied as a control group without any treatment and an experimental group in which etodolac, hydrazide and the derivative compounds were applied at increasing concentrations (10, 25, 50, 75, 100 µM). According to WST-8 colorimetric method, 3000 cells in 100 µl of medium per well were planted in 96-well plates. The plates were incubated overnight to allow the cells to adhere. The compounds were applied in 3 repetitions for the control and experimental groups. After the 24-hour incubation period, the medium was replaced with the fresh one and 10 µl of WST-8 was added to each well (Cell Counting Kit-8, KTC011001, Abbkine). The absorbance measurement was carried out at 450 nm after 4 hours by the using of a microplate reader (Synergy H1, BioTek Intruments Inc., USA). GraphPad Prism 8 program was used for statistical analysis.

4.2.2. Apoptosis studies

Determination of mitochondrial membrane potential

JC-1 mitochondrial membrane potential test was performed in PC-3, DU-145, LNCaP cell lines using compound **3b** with apoptotic potential according to its IC_{50} value, and etodolac as a control. This test is based on the principle that the JC-1 dye, which has the chemical structure [5,5,6,6-tetrachloro-1,1,3,3-tetraethylbenzimidazolylcarbocyanine iodide], enters to the mitochondria and gives different fluorescence intensity in healthy and apoptotic cells (JC-1 Mitochondrial Membrane Assay Kit, 10009172, Cayman Chemical). While J aggregates are formed in healthy cells to give red fluorescence, in apoptotic cells, JC-1 gives green fluorescence by remaining in its monomer structure. The depolarization of the cells due to apoptosis was determined according to the green/red fluorescence ratio. Based on this information, 50000 cells in 100 µl of medium per well were planted in 96-well black plates. To allow the cells to attach to the plates, they were incubated at 37°C, 5% CO_2 with RPMI-1640 medium overnight. After the medium was changed, **3b** and etodolac were prepared in different concentrations (10, 25, 50, 75, 100 µM) and applied to the cells. No treatment was applied for the control group. The medium was changed after 24 hours. JC-1 dye was diluted

1:9 in the medium. 5 µl of diluted JC-1 was added to each well and incubated for 30 minutes. Plates were centrifuged at 2100 rpm for 5 minutes. The supernatant was discarded. 150 µl of assay buffer was added to each well. Centrifugation and buffering was repeated once more. After centrifugation for the last time, 100 µl of assay buffer was added to each well. Fluorescence intensity for excitation and emission using a microplate reader was read at 535/595 nm for healthy cells and 485/535 nm for apoptotic cells, respectively.

Determination apoptosis with Annexin V/PI Staining

PC-3, DU145 and LNCaP prostate cancer cell lines were stained with Annexin V and propidium iodide (PI) in order to visualize apoptotic and necrotic cells (Tali Apoptosis Kit, A10788, Invitrogen). In apoptotic cells, Annexin V binds to phosphatidylserines translocated to the outer surface of the membrane and gives green fluorescence. In necrotic cells, however, the membrane integrity is lost, and PI enters the cell nucleus and gives red fluorescence. Based on this principle, cells were seeded in 6-well plates with 500 000 cells and 2 ml of RPMI-1640 medium in each well. The plates were incubated overnight at 37°C, 5% CO_2. The next day, the medium was replaced with fresh one and appropriate concentrations of drug were added. After 24 hours of incubation, cells were removed by trypsinization and transferred to eppendorfs. The eppendorfs were centrifuged at 2400 rpm for 5 minutes and the supernatant was discarded. 100 µl of 1X Annexin binding buffer and 5 µl of Annexin V were added to each eppendorf. After incubation for 20 minutes in the dark, they were centrifuged at 2400 rpm for 5 minutes and the supernatant was discarded. This time, 100 µl of 1X Annexin binding buffer and 1 µl of PI were added to each eppendorf and pipetted. Images of apoptotic and necrotic cells examined under fluorescence microscopy were captured on a computer after 5 minutes of incubation in the dark (CKX41, Olympus LS).

Acknowledgements: This study was not supported financially.

Author contributions: Concept – Ş.G.K., H.C.K.; Design – Ş.G.K., H.C.K.; Supervision – Ş.G.K.; Resources – Ş.G.K., H.C.K., İ.A., P.M.T., O.O.; Materials – Ş.G.K., H.C.K., İ.A., P.M.T., O.O.; Data Collection and/or Processing – Ş.G.K., H.C.K., İ.A., P.M.T., O.O.; Analysis and/or Interpretation – H.C.K., İ.A., P.M.T., O.O.; Literature Search – Ş.G.K., H.C.K., İ.A., P.M.T., O.O.; Writing – Ş.G.K., H.C.K., İ.A., P.M.T., O.O.; Critical Reviews – H.C.K., İ.A., P.M.T., O.O., Ş.G.K.

Conflict of interest statement: The authors declared no conflict of interest.

Appendix A. Supplementary Material

Supplementary material related to this article can be accessed at https://dx.doi.org/10.29228/jrp.97.

REFERENCES

[1] Kamijo T, Sato T, Nagatomi Y, Kitamura T. Induction of apoptosis by cyclooxygenase-2 inhibitors in prostate cancer cell lines. Int J Urol. 2001; 8(7): 35-39. [CrossRef]

[2] Shigemura K, Shirakawa T, Wada Y, Kamidono S, Fujisawa M, Gotoh A. Antitumor effects of etodolac, a selective cyclooxygenase-2 inhibitor, against human prostate cancer cell lines in vitro and in vivo. Urology. 2005; 66(6): 1239-1244. [CrossRef]

[3] Okamoto A, Shirakawa T, Bito T, Shigemura K, Hamada K, Gotoh A, Fujisawa M, Kawabata M. Etodolac, a selective cyclooxygenase-2 inhibitor, induces upregulation of e-cadherin and has antitumor effect on human bladder cancer cells in vitro and in vivo. Urology. 2008; 71(1): 156-160. [CrossRef]

[4] Çıkla P, Özsavcı D, Bingöl-Özakpınar Ö, Şener A, Çevik Ö, Özbaş-Turan S, Akbuğa J, Şahin F, Küçükgüzel ŞG. Synthesis, cytotoxicity, and pro-apoptosis activity of etodolac hydrazide derivatives as anticancer agents. Arch Pharm (Weinheim). 2013; 346(5): 367-379. [CrossRef]

[5] Çıkla-Süzgün P, Kaushik-Basu N, Basu A, Arora P, Talele TT, Durmaz I, Cetin-Atalay R, Kucukguzel SG. Anti-cancer and anti-hepatitis C virus NS5B polymerase activity of etodolac 1,2,4-triazoles. J Enzyme Inhib Med Chem. 2015; 30(5): 778-785. [CrossRef]

[6] Çoruh I, Çevik Ö, Yelekçi K, Djikic T, Küçükgüzel ŞG. Synthesis, anticancer activity, and molecular modeling of etodolac-thioether derivatives as potent methionine aminopeptidase (type II) inhibitors. Arch Pharm (Weinheim). 2018; 351(3-4): e1700195. [CrossRef]

[7] Rollas S, Küçükgüzel ŞG. Biological activities of hydrazone derivatives. Molecules. 2007; 12: 1910-1939. [CrossRef]

[8] Kömürcü ŞG, Rollas S, Ülgen M, Gorrod JW, Çevikbaş A. Evaluation of some arylhydrazones of p-aminobenzoic acid hydrazide as antimicrobial agents and their in-vitro hepatic microsomal metabolism. Boll Chim Farm. 1995; 134(7): 375-379.

[9] Koçyiğit-Kaymakçıoğlu B, Ünsalan S, Küçükgüzel ŞG, Şener G, Rollas S. HPLC analysis of in vivo metabolites of 4-nitrobenzoic acid [(5-nitro-2-thiophenyl)methylene]hydrazide in rats. Eur J Drug Metab Pharmacokinet. 2007; 32(4): 197-203. [CrossRef]

[10] Rollas S, Küçükgüzel ŞG. Hydrazone, amide, carbamate, macromolecular and other prodrugs of doxorubicin. Open Drug Deliv J. 2008; 2: 77-85. [CrossRef]

[11] Aydın S, Kaushik-Basu N, Arora P, Basu A, Nichols DB, Talele TT, Akkurt M, Çelik İ, Büyükgüngör O, Küçükgüzel ŞG. Microwave assisted synthesis of some novel flurbiprofen hydrazide-hydrazones as anti-HCV NS5B and anticancer agents. Marmara Pharm J. 2013; 17: 181-189. [CrossRef]

[12] Küçükgüzel ŞG, Koç D, Çıkla-Süzgün P, Özsavcı D, Bingöl-Özakpınar Ö, Mega-Tiber P, Orun O, Erzincan P, Sağ-Erdem S, Şahin F. Synthesis of tolmetin hydrazide-hydrazones and discovery of a potent apoptosis inducer in colon cancer cells. Arch Pharm (Weinheim). 2015; 348(10): 730-742. [CrossRef]

[13] Şenkardeş S, Kaushik-Basu N, Durmaz İ, Manvar D, Basu A, Atalay R, Küçükgüzel ŞG. Synthesis of novel diflunisal hydrazide-hydrazones as anti-hepatitis C virus agent and hepatocellular carcinoma inhibitors. Eur J Med Chem. 2016; 108: 301-308. [CrossRef]

[14] Çıkla-Süzgün P, Küçükgüzel ŞG. Recent advances in apoptosis: the role of hydrazones. Mini Rev Med Chem. 2019; 19(17): 1427-1442. [CrossRef]

[15] Han Mİ, Bekçi H, Uba Aİ, Yıldırım Y, Karasulu E, Cumaoğlu A, Karasulu HY, Yelekçi K, Yılmaz Ö, Küçükgüzel ŞG.Synthesis, molecular modeling, in vivo study, and anticancer activity of 1,2,4-triazole containing hydrazide-hydrazones derived from (S)-naproxen. Arch Pharm (Weinheim). 2019; 352(6): e1800365. [CrossRef]

[16] Han Mİ, Atalay P, İmamoğlu N, Küçükgüzel ŞG. Synthesis, characterization and anticancer activity of novel hydrazide-hydrazones derived from ethyl paraben. J Res Pharm. 2020; 24(3): 341-349. [CrossRef]

[17] Şenkardeş S, Türe A, Ekrek S, Durak AT, Abbak M, Çevik Ö, Kaşkatepe B, Küçükgüzel İ, Küçükgüzel ŞG. Novel 2,6-disubstituted pyridine hydrazones: synthesis, anticancer activity, docking studies and effects on caspase-3-mediated apoptosis. J Mol Struct. 2021; 1223: 128962. [CrossRef]

[18] Han Mİ, Atalay P, Tunç CÜ, Ünal G, Dayan S, Aydın Ö, Küçükgüzel ŞG. Design and synthesis of novel (S)-naproxen hydrazide-hydrazones as potent VEGFR-2 inhibitors and their evaluation in vitro/in vivo breast cancer models. Bioorg Med Chem. 2021; 37: 116097. [CrossRef]

[19] Rutavichyus A, Valiulene S, Kuodis Z. Isomerism of hydrazones of (2-benzothiazolylthio)- and (2-benzoxazolylthio)acetic acids. Chem Heterocycl Comp. 1995; 31(5): 629-633. [CrossRef]

[20] Rutavichyus A, Valiulene S, Kuodis Z. Synthesis and structure of hydrazones obtained from hydrazides of [5-(4-pyridyl)-1,3,4-oxadiazol-2-ylthio]acetic or 2-[5-(4-pyridyl)-1,3,4-oxadiazol-2-ylthio]propionic acids. Chem Heterocycl Comp. 2000; 36(7): 851-856. [CrossRef]

[21] Çıkla P, Tatar E, Küçükgüzel İ, Şahin F, Yurdakul D, Basu A, Krishnan R, Nichols DB, Kaushik-Basu N, Küçükgüzel ŞG. Synthesis and characterization of flurbiprofen hydrazide derivatives as potential anti-HCV, anticancer and antimicrobial agents. Med Chem Res. 2013; 22: 5685-5699. [CrossRef]

[22] Vincenzo G, Franco P. 3-(2-trialkylsilyloxy)ethyl-7-ethyl-1H-indoles and method for their preparation. US 5,599,946. 1997.

[23] Raghavan BV, Ramana KV, Khera B, Kumar N. Process for the preparation of 1,8-disubstituted-1,3,4,9-tetrahydropyrano (3,4-b)-indole-1-acetic acid esters. US 6,331,638 B1. 2001.