Egypt. J. Chem. **Vol. 63,** No. 11 pp. 4175 - 4184 (2020)

Egyptian Journal of Chemistry

http://ejchem.journals.ekb.eg/

Design, Synthesis and Biological Evaluation of Novel Indole-Thalidomide Hybrids Analogs

M. A.-H. Zahran[1]*, S. EL Kosey[1], A. B. Mehany[2], M. H. Gebreil[1]

[1] *Faculty of Science, Department of Chemistry, Menoufia University, Shebin El-Koom, Egypt*

[2] *Faculty of Science, Department of Zoology, Al-Azhar University, Cairo, Egypt*

Abstract

A series of novel Indole-Thalidomide hybrids were designed and synthesized in a good yield to improve and develop higher potency and selective anti-tumor agents. By two steps alkylation of Indole with Thalidomide then react the product with different active methylene groups. Their anti-proliferative evaluation activities against HCT 116 (colon cancer cell line), HepG2 (liver cancer cell line) and MCF-7 (breast cancer cell line). In vitro were tested by using Sulforhodamine-B stain (SRB) assay. The chemical structures of all synthesized analogs were elucidated on the basis of their spectral IR, ^{1}H NMR, ^{13}C NMR, Mass spectroscopy and element analysis. The result of the present work indicated all new analogs showed good activity toward all the tested cell lines, analog **8** has broad-spectrum anticancer activity toward all the tested cancer cell lines, followed by analog **2**. The apoptosis evaluation for new analogs on Caspase-**3**, Bcl2 and Bax shown the best activity for analogs **2** and **8**, so that the two analogs were histopathologically investigated.

Keywords: Indole, Thalidomide, Knoevenagel condensation, L-proline, HCT116, Caspes-3, Histopathological.

1. Introduction

Cancer is one of the largest causes of death, it is characterized by the uncontrolled growth and spread of abnormal cells. It is caused by a lot of factor that named carcinogenic factors such as smoking, infectious organisms, inherited genetic mutations, hormones, and immune conditions. Treatments include surgery, chemotherapy, radiation, hormone, and immune therapy **[1]**. New global cancer data suggests that the global cancer burden has risen to 18.1 million cases and 9.6 million cancer deaths **[2]**. Therefore, the research for new cancer-treating agents is an important area in both organic and medicinal chemistry. Indoles are the main components of many drugs and some biologically active compounds, and the development of new forming methods leading to indole derivatives has attracted much attention in organic synthesis **[3]**. Indole derivatives have been reported to possess a wide variety of biological and pharmacological properties **[4-19]**. Isoindole-1,3-dione is known as Phthalimide which researcher focus to evaluate biological activity **[20-24]**, also when linked to piperdie-2,6-dione to form [(R,S)2-(2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione] (Thalidomide- racemic) which known as a drug and have antiangiogenic, immunomodulatory and significant antitumor activities also its analogs showed significant potency against several diseases such as prostate cancer, HIV-related ulcers, multiple myeloma, and Kaposi's sarcoma **[25-35**, **46-48]**. Recently, our group synthesizes novel indole, phthalimide, thalidomide analogs and studies intensively their antitumor activity **[36-45]**. Based on the previously mentioned facts, the current study aimed to synthesize novel Thalidomide–Indole hybrids by Knoevenagel condensation between 1H-indole-3-carbaldehyde and different active methylene Compounds. The obtained products were evaluated and investigated against different cancer cell lines.

2. Experimental

Materials and methods

Melting points for the synthesized compounds were measured using a Stuart melting point apparatus. The reaction progress was monitored using

*Corresponding author e-mail: magdyzahran@gmail.com , (+2010072080275).
Receive Date: 08 February 2020, Revise Date: 10 March 2020, Accept Date: 11 March 2020
DOI: 10.21608/EJCHEM.2020.23685.2408

TLC aluminum sheets silica gel (Merck60F254) and visualized using UV lamp. IR were recorded for 1, 2, 8 and 9 by FTIR 4100 Jasco – Japan and for 3, 4, 5, 6 and 7 by FT-IR perkinElmer. All ^{1}H and ^{13}CNMR experiments (solvent DMSO-*d*6) were carried out with a 300 and 500 MHz varian and Bruker Avance at the main chemical laboratories, (Cairo and Mansoura University) Egypt. Chemical shifts are reported in part per million (ppm) relative to the respective solvent or tetramethylsilane (TMS). Mass spectra were recorded with GC-2010 Shimadzu spectrometer in EI (70 eV) mode. The elemental analyses were performed by Vario EL M Germany at the Micro Analytical Center, Cairo University, Egypt. All chemicals and solvents were purchased from E. Merck (Darmstadt, Germany) and Sigma Aldrich, thalidomide analog 2-(1-(chloromethyl)-2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione **[20]** and compound 3-methyl-1H-pyrazol-5(4H)-one **[19]** were prepared in laboratory according to the reported methedology. The biological activity analysis was carried out in central laboratory, Faculty of Science, Al- Azhar University, Egypt.

General procedure for the synthesis of 1-((3-(1,3-dioxoisoindolin-2-yl)-2,6-dioxopiperidin-1-yl)methyl)-1H-indole-3-carbaldehyde (1)

To a solution of 1H-indole-3-carbaldehyde (1 mmol, 0.145 gm) in dry DMF (5 ml), potassium carbonate (1 mmol, 0.138 gm) was added, and kept stirring at room temperature overnight, then 2-(1-(chloromethyl)-2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione (1 mmol, 0.306 gm) was added slowly. The reaction was monitored by TLC after 2 h under stirring at room temperature, pour mixture over ice distilled water and leave to stirring for one hour then filtrate. The precipitate was collected and crystallized from ethanol. White Solid; m.p. 260–262°C; yield 70%; IR (KBr) ν cm^{-1}: 1715 (C=O, aldhyde), 1692,1667 (C=O); ^{1}H NMR (300 MHz, DMSO-*d*6):δ 9.95 (s, 1H, CHO), 8.29(s, 1H, H-2" $_{Indole}$), 8.09 (m, 1H, H $_{Arom\ (Indole)}$), 7.92 (m, 4H, H $_{Arom}$), 7.76 (m, 1H, H $_{Arom\ (Indole)}$), 7.29 (m, 2H, H$_{Arom\ (Indole)}$), 6.14 (s, 2H, NCH_2N), 5.38 (m, 1H, H-3'), 2.78 (m, 4H, $C_{4',5'}$ - H); ^{13}C NMR (75 MHz, DMSO-*d*6): δ 185.326 (CHO), 171.971, 170.066 ($C_{2'}$, $C_{6'}$), 166.989 (C_1 , C_3), 142.09 ($C_{2"}$), 136.87 ($C_{8"}$), 134.90 (C_5 , C_6), 131.14 (C_{3a} ,C_{7a}), 124.16 ($C_{9"}$), 123.78(C_4 , C_7), 122.66 ($C_{4"}$), 120.78 ($C_{6"}$), 120.39 ($C_{5"}$), 117.75 ($C_{3"}$), 111.63 ($C_{7"}$), 49.74 (N-C-N), 49.49 ($C_{3'}$), 31.15 ($C_{5'}$), 20.79 ($C_{4'}$); MS: m/z (%) : 415.25 ($[M]^+$, 93.59), (MW 415.41 g mol^{-1}); Anal.calcd. for $C_{23}H_{17}N_3O_5$: C, 66.5; H, 4.12; N, 10.12. Found: C, 66.34; H, 4.26; N, 10.27.

General procedure for synthesis compound (2-9):

To a solution of synthesis Indole-Thalidomide hybrid (compound 1) (1 mmol, 0.415 gm) in absolute ethanol (8 ml), L-proline (0.2 mmol, 0.03 gm) was added as a catalyst, and then the appropriate active methylene compounds (2 mmol) was added. The reaction mixture was heated under reflux at 60°C. The reaction was monitored by TLC after 3 h. the precipitation was collected, washed and crystallized from ethanol scheme (1).

Ethyl(E)-2-cyano-3-(1-((3-(1,3-dioxoisoindolin-2-yl)-2,6-dioxopiperidin-1-yl)methyl)-1H-indol-3-yl)acrylate (2).

Yellow Solid; m.p. 270–272°C; yield 75%; IR (KBr) ν cm^{-1}: 2213 (C≡N), 1716-1590 (C=O); ^{1}H NMR (300 MHz, DMSO-*d6*): δ 8.76 (s, 1H, H-2" $_{Indole}$), 8.52 (s, 1H, CH), 7.88 (m, 6H, H $_{Arom}$), 7.32 (m, 2H, H $_{Arom}$), 6.20 (m, 2H, NCH_2N), 5.38 (m, 1H, H-3'), 4.29 (m, 2H, $CH_{2\ ester}$), 2.84 (m, 4H, $C_{4',5'}$ - H), 1.30 (t, *J*=6.6 Hz, 3H, $CH_{3\ ester}$);^{13}C NMR (75 MHz, DMSO-*d*6): δ 171.91, 169.95 ($C_{2'}$, $C_{6'}$), 166.84 (C_1 , C_3), 162.80 (C=O ester), 145.47 (CH), 136.37 ($C_{2"}$), 136.02 ($C_{8"}$), 134.77 (C_5 , C_6), 131.08 (C_{3a} , C_{7a}), 126.92 (C_4 , C_7), 123.81 ($C_{9"}$), 123.34 ($C_{6"}$), 122.46 ($C_{5"}$), 118.39 ($C_{4"}$), 117.10 (CN), 111.93 ($C_{3"}$), 109.71 ($C_{7"}$), 94.22 (tert C $_{Aliph}$), 61.46 (N-C-N), 49.96 (CH_2 $_{ester}$), 49.44, 31.07, 20.76 ($C_{3'}$, $C_{5'}$, $C_{4'}$), 13.980 (CH_3 $_{ester}$); MS: m/z (%): 509.80 ($[M-1]^+$, 100), (MW 510.51 g mol^{-1}); Anal.calcd. for $C_{28}H_{22}N_4O_6$: C, 65.88; H, 4.34; N, 10.97. Found: C, 65.70; H, 4.45; N, 10.86.

2-((1-((3-(1,3-dioxoisoindolin-2-yl)-2,6-dioxopiperidin-1-yl)methyl)-1H-indol-3-yl)methylene)malononitrile (3).

Pale yellow Solid; m.p. 225 - 227°C; yield 70%; IR (KBr) ν cm^{-1} 2242, 2223 (2 C≡N), 1785-1697 (C=O); ^{1}H NMR (300 MHz, DMSO-*d*6): δ. 8.61 (s, 1H, H-2" $_{Indole}$), 8.37 (s, 1H, CH), 8.16 (m, 1H, H $_{Arom}$), 7.94 (m, 4H, H $_{Arom}$), 7.79 (m, 1H, H $_{Arom}$), 7.32 (m, 2H, H $_{Arom}$), 6.19 (s, 2H, NCH_2N), 5.32 (m, 1H, H-3' $_{piperidine}$), 2.80 (m, 4H, $C_{4',5'}$ - H $_{piperidine}$); ^{13}C NMR (75 MHz, DMSO-*d*6): δ 171.72, 169.09 ($C_{2'}$, $C_{6'}$), 166.13 (C_1 , C_3), 152.38 (CH), 142.09, 136.86 ($C_{8"}$, $C_{2"}$), 134.91, 131.17 (C_5 , C_6 , C_{3a} , C_{7a}), 124.14 ($C_{9"}$), 123.77 (C_4 , C_7), 123.45, 122.65, 120.78 ($C_{6"}$, $C_{5"}$, $C_{4"}$), 120.39 (2 x CN), 116.75, 108.55 ($C_{3"}$, $C_{7"}$), 69.21 (tert C $_{Aliph}$), 48.43 (N-C-N), 48.11, 31.90, 21.88 (piper. $C_{3'}$, $C_{4'}$, $C_{5'}$); MS: m/z (%): 463.25 ($[M]^+$, 36.33), (MW 463.45 g mol^{-1}); Anal.calcd. for $C_{26}H_{17}N_5O_4$: C, 67.38; H, 3.70; N, 15.11. Found: C, 67.65; H, 3.57; N, 15.03.

Dimethyl2-((1-((3-(1,3-dioxoisoindolin-2-yl)-2,6-dioxopiperidin-1-yl)methyl)-1H-indol-3-yl)methylene)malonate (4):

White Solid; m.p. 230°C; yield 69%; IR (KBr) ν cm^{-1}:1718,1691 (C=O); ^{1}H NMR (500 MHz, DMSO-

*d*6): δ 7.91 (m, 6H, H $_{Arom}$), 7.75 (m, 2H, H $_{Arom}$), 7.24 (m, 2H, CH , H-2"), 6.10 (m, 2H, N-CH_2-N), 5.35 (m, 1H, H-3' $_{piperidine}$), 3.75 (s, 6H, 2 x CH_3), 2.86 (m, 4H, $C_{4',5'}$ - H $_{piperidine}$); ^{13}C NMR (125 MHz, DMSO-*d*6): δ 172.16, 170.23 ($C_{2'}$, $C_{6'}$), 167.25 (C_1 , C_3), 167.09 (2 x C=O $_{ester}$), 164.47 (CH), 136.05, 135.02 ($C_{8"}$, $C_{2"}$), 133.47 (tert C $_{Aliph}$), 132.94 (C_5 , C_6), 131.21 (C_{3a} , C_{7a}), 126.97 ($C_{9"}$), 123.54 (C_4 , C_7), 123.25, 121.73, 119.28, 118.09, 111.73 ($C_{3"}$,$C_{4"}$, $C_{5"}$, $C_{6"}$, $C_{7"}$), 52.28 (2 x CH_3), 49.61(N-C-N), 49.52 , 31.20, 20.81 ($C_{3'}$, $C_{4'}$, $C_{5'}$); MS m/z (%): 529.15 ([M]$^+$, 100), (MW 529.51g mol^{-1});Anal.calcd. for $C_{28}H_{23}N_3O_8$: C, 63.51; H, 4.38; N, 7.94 Found: C, 63.39; H, 4.51; N, 7.75.

Diethyl2-((1-((3-(1,3-dioxoisoindolin-2-yl)-2,6-dioxopiperidin-1-yl)methyl)-1H-indol-3-yl)methylene)malonate (5):

Pale yellow Solid; m.p. 243- 245°C; yield 77%; IR (KBr) ν cm^{-1}:1784,1669 (C=O); 1H NMR (300 MHz, DMSO-*d*6): δ 7.94 (s, 2H, CH & H-2" $_{Indole}$), 7.90 (m, 4H, H $_{Arom}$), 7.75 (m, 2H, H $_{Arom}$), 7.25 (m, 2H, H $_{Arom}$), 6.09 (s, 2H, N-CH_2-N), 5.36 (m, 1H, H-3' $_{piperidine}$), 4.26 (m, 2H, CH_2 $_{ester}$), 4.22 (m, 2H, CH_2 $_{ester}$), 2.80 (m, 4H, $C_{4',5'}$ - H $_{piperidine}$), 1.25 (t, *J*=6.9Hz, 3H, CH_3 $_{ester}$), 1.16 (t, *J*=7.2Hz, 3H, CH_3 $_{ester}$); ^{13}C NMR (125 MHz, DMSO-*d*6): δ 172.14, 170.22 ($C_{2'}$, $C_{6'}$), 167.10 (C_1 , C_3), 166.87 (2 x C=O $_{ester}$), 164.09 (CH), 136.03, 135.01, 132.88, 132.61 (C_5 , C_6 , $C_{2"}$, $C_{8"}$), 131.22 (tert C $_{Aliph}$), 127.03 (C_{3a} , C_{7a}), 123.54 ($C_{9"}$), 123.23 (C_4 , C_7), 121.68, 120.04, 118.06, 111.72, 108.91 ($C_{3"}$, $C_{4"}$, $C_{5"}$, $C_{6"}$, $C_{7"}$), 61.305 (N-C-N), 60.87 ($C_{3'}$), 49.66 , 49.53 (2 x CH_2 $_{ester}$), 31.23, 20.82 ($C_{4'}$, $C_{5'}$), 14.13, 13.65 (2 x CH_3 $_{ester}$) ; MS: m/z (%): 557.55 ([M]$^+$, 100), (MW 557.56 g mol^{-1}) ; Anal.calcd. for $C_{30}H_{27}N_3O_8$: C, 64.63; H, 4.88; N, 7.54. Found: C, 64.47; H, 4.74; N, 7.65.

Ethyl(Z)-3-(1-((3-(1,3-dioxoisoindolin-2-yl)-2,6-dioxopiperidin-1-yl)methyl)-1H-indol-3-yl)-2-(methylthio)acrylate (6):

White Solid; m.p. 250 - 252°C; yield 76%; IR (KBr) ν cm^{-1}: 1773-1624 (C=O); ^{1}H NMR (300 MHz, DMSO-d6): δ 11.10 (s, 1H, CH), 8.58 (s, 1H, H-2" $_{Indole}$), 7.95 (m, 6H, H A $_{Arom}$), 7.23 (m, 2H, H $_{Arom}$), 6.14 (s, 2H, N-CH_2-N), 5.40 (m, 1H, H-3' $_{piperidine}$), 4.25 (q, *J*=6.9Hz, 2H, CH_2 $_{ester}$), 2.69 (m, 4H, $C_{4',5'}$ - H $_{piperidine}$), 2.31 (s, 3H, SCH_3), 1.31 (t, *J*=6.9Hz, 3H, CH_3 $_{ester}$) ; ^{13}C NMR (125 MHz, DMSO-*d*6): δ 172.83, 172.12 ($C_{2'}$, $C_{6'}$), 170.21 (C_1 , C_3), 169.91(C=O $_{ester}$), 167.20, 167.07 ($C_{2"}$, $C_{8"}$), 165.55 (tert C $_{Aliph}$), 135.56, 135.02 (C_5 , C_6 , C_{3a} , C_{7a}), 134.94 ($C_{9"}$), 134.18 (CH), 131.25 (C_4 , C_7), 131.23, 123.56, 123.46,111.52, 110.57 ($C_{3"}$, $C_{4"}$, $C_{5"}$, $C_{6"}$, $C_{7"}$), 61.03 (N-C-N), 60.86 ($C_{3'}$), 49.54 (CH_2 $_{ester}$), 30.96, 22.01 ($C_{4'}$, $C_{5'}$),16.76 (SCH_3), 14.25 (CH_3 $_{ester}$) ; MS: m/z (%): 531.50 ([M]$^+$, 100), (MW 531.58 g mol^{-1}) ; Anal.calcd. for $C_{28}H_{25}N_3O_6S$: C, 63.27; H, 4.74; N, 7.90. Found: C, 63.36; H, 4.88; N, 7.76.

Ethyl(E)-2-((1-((3-(1,3-dioxoisoindolin-2-yl)-2,6-dioxopiperidin-1-yl)methyl)-1H-indol-3-yl)methylene)-3-oxobutanoate (7):

White Solid; m.p. 225 - 226°C; yield 65%; IR (KBr) ν cm^{-1}: 1783-1622 (C=O); ^{1}H NMR (500 MHz, DMSO-*d*6): δ 7.89 (m, 6H, H $_{Arom}$), 7.80 (s, 1H, CH), 7.75 (m, 1H, H-2" $_{Indole}$), 7.25 (m, 2H, H $_{Arom}$), 6.08 (m, 2H, N-CH_2-N), 5.35 (m, 1H, H-3' $_{piperidine}$), 4.21 (q, *J*=7Hz, 2H, CH_2 $_{ester}$), 2.60 (m, 4H, $C_{4',5'}$ - H $_{piperidine}$), 2.42 (s, 3H, CH_3 $_{ketone}$), 1.19 (t, *J*=7Hz, 3H, CH_3 $_{ester}$) ; ^{13}C NMR (125 MHz, DMSO-*d*6): δ 195.18 (C=O $_{ketone}$), 172.14, 170.21 ($C_{2'}$, $C_{6'}$), 168.12 (C=O $_{ester}$), 167.09 (C_1 , C_3), 135.00 (tert C $_{Aliph}$), 132.65 (CH), 132.52, 131.21 ($C_{8"}$, $C_{2"}$), 130.10, 123.53 (C_5 , C_6 , C_{3a} , C_{7a}), 123.26 ($C_{4"}$, $C_{9"}$), 121.50 (C_4 , C_7), 111.65, 109.30 ($C_{3"}$, $C_{5"}$, $C_{6"}$, $C_{7"}$), 61.10 (N-C-N), 60.75 ($C_{3'}$), 49.52 (CH_2 $_{ester}$), 31.23 ($C_{5'}$), 25.77 (CH_3 $_{ketone}$), 20.81 ($C_{4'}$), 14.01 (CH_3 $_{ester}$); MS: m/z (%): 527.15 ([M]$^+$, 100), (MW 527.53 g mol^{-1}) ; Anal.calcd. for $C_{29}H_{25}N_3O_7$: C, 66.03; H, 4.78; N, 7.97. Found: C, 66.23; H, 4.94; N, 7.81.

2-(1-((3-((4,6-dioxo-2-thioxotetrahydropyrimidin-5(2H)-ylidene)methyl)-1H-indol-1-yl)methyl)-2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione (8):

Dark yellow Solid; m.p. 295°C; yield 80%; IR (KBr) ν cm^{-1}: 3429, 3425 (2 N-H), 1775-1664 (C=O); ^{1}H NMR (300 MHz, DMSO-*d*6): δ 12.24 (s, 1H, NH $_{pyrimidine}$), 12.18 (s, 1H, NH $_{pyrimidine}$), 9.78 (s, 1H, CH), 8.65 (s, 1H, H-2" $_{Indole}$), 7.91 (m, 6H, H $_{Arom}$), 7.37 (m, 2H, H $_{Arom}$), 6.23 (s, 2H, N-CH_2-N), 5.36 (m, 1H, H-3' $_{piperidine}$), 2.76 (m, 4H, $C_{4',5'}$ - H $_{piperidine}$) ; ^{13}C NMR (125 MHz, DMSO-*d*6): δ 177.83 (C=S), 172.06, 170.14 ($C_{2'}$, $C_{6'}$), 167.04 (2 x C=O $_{pyrimidine}$), 162.58 (C_1 , C_3), 160.78 (CH), 144.89, 143.84 ($C_{2"}$, $C_{8"}$), 136.51 (tert C $_{pyrimidine}$), 135.00, 131.20 (C_5 , C_6 , C_{3a} , C_{7a}), 129.08 ($C_{9"}$), 124.19 (C_4 , C_7), 123.57, 123.38, 117.79, 111.70, 110.25 ($C_{3"}$, $C_{4"}$, $C_{5"}$, $C_{6"}$, $C_{7"}$), 50.39 ($C_{3'}$), 49.52 (N-C-N), 31.24, 20.84 ($C_{4'}$, $C_{5'}$); MS: m/z (%): 540.80 ([M-1]$^+$, 17.99), (MW 541.54 g mol^{-1}) ; Anal.calcd. for $C_{27}H_{19}N_5O_6S$: C, 59.88; H, 3.54; N, 12.93. Found: C, 59.96; H, 3.63; N, 12.79.

(E)-2-(1-((3-((3-methyl-5-oxo-1,5-dihydro-4H-pyrazol-4-ylidene)methyl)-1H-indol-1-yl)methyl)-2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione (9):

Yellow Solid; m.p. 285°C; yield 70%; IR (KBr) ν cm^{-1}: 3398 (N-H), 1774-1600 (C=O); ^{1}H NMR (300 MHz, DMSO-*d*6): δ 10.97 (s, 1H, NH $_{pyrazole}$), 9.90 (s, 1H, CH), 8.09 (m, 1H, H-2" $_{Indole}$), 7.62 (m, 8H, H $_{Arom}$), 6.17 (m, 2H, N-CH_2-N), 5.35 (m, 1H, H-3' $_{piperidine}$), 2.55 (m, 4H, $C_{4',5'}$ - H $_{piperidine}$), 2.49 (m, 3H,

CH_3); ^{13}C NMR (125 MHz, DMSO-*d*6): δ 171.96 (C=O $_{pyrazole}$), 170.01, 167.04, 166.10 ($C_{2'}$, $C_{6'}$, C_1, C_3), 149.37 ($C_{3'''\ pyrazole}$), 140.87 (CH), 136.10 ($C_{8''}$), 134.98 (C_5 , C_6), 134.10 ($C_{2''}$), 131.20 (C_{3a} , C_{7a} , $C_{4'''\ pyrazole}$), 128.29 ($C_{9''}$), 123.54 (C_4 , C_7), 122.14, 120.34, 118.56, 111.92, 111.55 ($C_{3''}$, $C_{4''}$, $C_{5''}$, $C_{6''}$, $C_{7''}$), 50.30 (N-C-N), 49.54, 31.23, 20.87 ($C_{3'}$, $C_{4'}$, $C_{5'}$), 13.02 (CH_3); MS: m/z (%): 494.80 ($[M-1]^+$, 100), (MW 495.50 g mol^{-1}); Anal.calcd. for $C_{27}H_{21}N_5O_5$: C, 65.45; H, 4.27; N, 14.13. Found: C, 65.52; H, 4.32; N, 14.02.

Cytotoxic activity

1-Materials and methods:

Chemicals: All chemicals used in this study are high analytical grade. They were obtained from (either Sigma-Alderich or Biorad).

Human tumor cell lines: The tumor cell lines were obtained frozen in liquid nitrogen (-180 °C) from the American Type Culture Collection (ATCC) and was maintained at the National Cancer Institute, Cairo, Egypt, by serial subculturing.

2-Measurement of potential cytotoxic activity:

The cytotoxic activity was measured in vitro on human cancer cell line HEPG2, MCF7 and HCT116 using Sulforhodamine-B stain (SRB) assay applying the method of Skehan, et al **[49]**. Cells were plated in 96 multiwell plates for 24 hours before treatment with the analogs to allow attachment of the cells to the wall of the plate. Different concentrations of the compound under test (0, 6.25, 12.5, 25, 50 and 100 µg/mL) were added to the cell monolayer. Triplicate wells were prepared for each individual dose.Monolayer cells were incubated with the analogs for 48 hours at 37 °C and in atmosphere of 5% CO2. After 48 hours cell was fixed, washed and stained with Sulforhodamine B stain. Excess stain was washed with acetic acid and attached stain was recovered with Tris EDTA buffer. Colour intensity was measured in an ELISA reader. The relation between surviving fraction and drug concentration was plotted and IC_{50} (the concentration required for 50% inhibition of cell viability) was calculated for each analog by Sigmaplot software.

Caspase-3 & Bax and Bcl-2 evaluation

Cells were obtained from American Type Culture Collection, grown in RPMI 1640 containing 10% fetal bovine serum at 37°C, stimulated with the analogs to be tested for Bcl2, Bax, Caspase-3 and lysate with Cell Extraction Buffer. This lysate was diluted in Standard Diluent Buffer over the range of the assay and measured for active Bcl2, Bax, Caspase-3 content. (cells are Plated in a density of 1.2 – 1.8 × 10,000 cells/well in a volume of 100µl complete growth medium + IC_{50} of the tested compound for 24 hours before the enzyme assay).

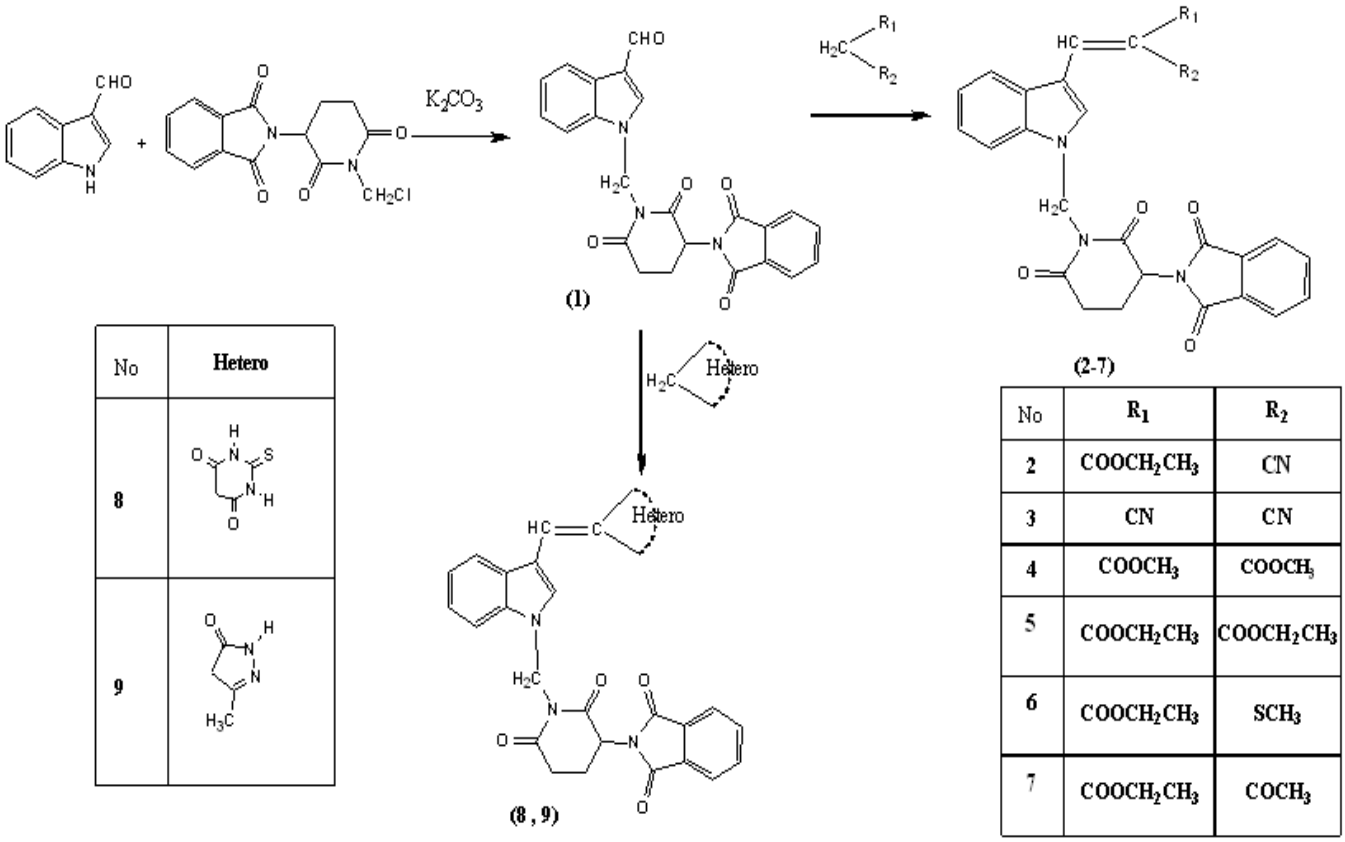
Histopathological

Cells were obtained from American Type Culture Collection, grown in RPMI 1640 containing 10% fetal bovine serum at 37°C and stimulated with the compounds 2 & 8 to be tested. After the treatment, the selected compounds and the cells have been harvest with trypsin and collect in falcon tube then centrifugation at 1200 rb. Cells were distributed on slides and fixed with absolute ethanol, then stained with Hematoxylin and Eosin **[50]**. The Stained sections were examined for circulatory disturbances, inflammation, degenerations, apoptosis, necrosis, and any other pathological changes in the examined tissues.

3. Results and Discussion

Chemistry.

The current study includes the synthesis of Indole – Thalidomide hybride (1) by alkylation of indole-3-carbaldehyde at N1 with 2-(1-(chloromethyl)-2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione under basic condition using potassium carbonate. The hybride resulting product (1) was submitted to react with different interesting active methylene compounds in presence of L-proline as a catalyst. The structures prove that the reaction takes place under knoevenagel condensation mechanism as shown in (scheme 1).



No	Hetero
8	
9	

No	R_1	R_2
2	$COOCH_2CH_3$	CN
3	CN	CN
4	$COOCH_3$	$COOCH_3$
5	$COOCH_2CH_3$	$COOCH_2CH_3$
6	$COOCH_2CH_3$	SCH_3
7	$COOCH_2CH_3$	$COCH_3$

Scheme 1: Alkylation of 1H-indole-3-carbaldehyde with 2-(1-(chloromethyl)-2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione then condensation with different active methylene group.

The resulting products (1-9) were elucidated with all spectroscopical tools IR, ^{1}HNMR, ^{13}C NMR, mass and elemental analysis.

The IR spectra of analogs (1-9) showed absorption (1600-1785 cm^{-1}) due to presence of C=O function, analog 2 give absorption peak at 2213 cm^{-1} due to presence of nitril group (CN), analog 3 showed absorption peak at 2242 cm^{-1} for two nitrilgroups (CN), analog 8 showed peak with two spikes at (3429, 3425 cm^{-1}) for two imine groups (NH) while for analog 9 showed peak with one spike 3398 cm^{-1} for imine group (NH).

The ^{1}H NMR spectrum of analog 1 showed a singlet signal at 9.95 ppm for aldehydic proton (CHO) and a singlet signal at 6.14 ppm for methylene group (N-CH_2-N), analog 2 showed peak at 4.29 for (CH_2) and triplet peak at 1.30 for (CH_3),analog 4 showed two singlet peaks at 3.752, 3.756 ppm for two methyl groups (CH_3), analog 5 showed two triplet signals at 1.16 ppm & 1.25 ppm for two methylgroups (CH_3), analog 6 showed singlet peak at 3.30 ppm for mercaptomethyl group (SCH_3), analog 7 showed singlet peak at 3.34 ppm for acetyl group ($COCH_3$).

BIOLOGICAL ACTIVITIES.

Antiproliferative activity

All synthesized analogs were tested against HCT 116, HepG2 and MCF-7 cells using Sulforhodamine-B stain (SRB) assay and Thalidomide as reference drug. IC_{50} values were reported in Table 1. All analogs show good activity on HCT 116, HepG2 and MCF-7. IC_{50} value of HCT-116 for analogs in range (9.74 – 39.59 µM) comparing with Thalidomide (55.73 µM), IC_{50} value of HEPG-2 for new analogs in range (8.92 – 35.51 µM) comparing with Thalidomide (50.51 µM), IC_{50} value of MCF-7 for new analogs in range (12.5 – 43.64 µM) comparing with Thalidomide (58.72 µM) and the result of analog 8 & 2 shown the highest potency. The results lead to further histopathological investigations in order to clarify the potent activity of both analogs.

Table 1: Anticancer activity of Thalidomide analogs on different human cancer cell lines.

Analog	IC_{50} (µM)		
	HCT 116	HEPG-2	MCF-7
1	17.52	15.71	20.02
2	11.94	10.51	13.56
3	24.98	20.73	28.09
4	15.61	13.76	20.13
5	22.64	19.73	26.58
6	18.91	15.92	31.81
7	28.51	24.32	31.21
8	9.74	8.92	12.5
9	39.59	35.51	43.64
Thalidomide (reference drug)	55.73	50.51	58.72

Caspase-3 Activation Assay

The cysteine aspartase group, namely caspases, plays a crucial role in the induction of apoptosis and amongst them, caspase-3 happens to be one of the effective caspases. Hence, the synthesized analogs were investigated against caspase-3. The results indicate that all analogs induced caspase-3. The most active derivatives were 2 and 8 analogs which showed a highly activation as reference drug (Lapatinib) and eleven times more than the control (cell free). Other analogs shown a good activation but less than reference drug and three to nine folds effective than the control. This means that these analogs possess potent apoptotic activity for cancer cell lines as shown in Table 2& Fig.1.

Table 2: Caspes-3 activation assay for analogs compare with reference drug (Lapatinib).

Analogs	Casp3 conc. pg/mL	FLD
1	489.41	9.92
2	526.83	10.67
3	370.80	7.51
4	468.04	9.48
5	310.47	6.29
6	421.97	8.55
7	245.37	4.97
8	546.45	11.07
9	187.26	3.79
Reference (Lapatinib)	563.22	11.41
Control (cell free)	49.36	1

Bax Evaluation

Further investigation for all the synthesized analogs were tested for Bax which is considered as a pro-apoptotic gene and one of apoptosis marker. Observed data demonstrate that all analogs have ability to up regulate Bax expression from three to nine times more than control and two of analogs 2 and 8 up regulate Bax nine times such as reference drug (Lapatinib) as shown in Table 3& Fig.1.

Table 3: Bax evaluation for HepG2 with analogs and reference drug (Lapatinib).

BAX HepG2		
Analogs	conc. Pg/mL	FLD
1	304.5	7.60
2	321.6	8.03
3	201.9	5.04
4	246.7	6.16
5	185.3	4.63
6	219.4	5.48
7	162.8	4.06
8	370.8	9.26
9	122.6	3.06
Reference (Lapatinib)	378.5	9.45
Control (cell free)	40.06	1

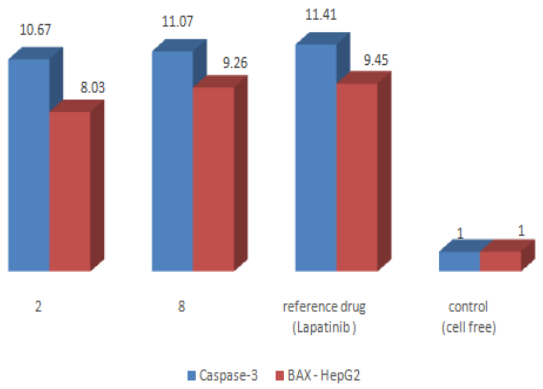


Fig.1most potent active analogs and reference drug

Bcl-2 Evaluation

On the other hand, analogs (1-9) were tested for Bcl-2, one of Bcl-2 family is responsible for apoptosis which represent the anti apoptotic gene and down regulation of Bcl-2 which means inducing cells for apoptosis. Data showed that all analogs possess a good down regulation for Bcl-2 from 0.2 to 0.6 folds comparable to the control and one of analogs down regulate Bcl-2 more less than reference drug (Lapatinib) this means that this analog 8 possess inducers for apoptosis as shown in Table 4 & Fig.2.

Table 4: BCl2 evaluation for analogs and reference drug (Lapatinib).

Bcl2 for HepG2		
Analogs	conc. Pg/mL	FLD
1	2.38	0.43
2	2.10	0.38
3	3.05	0.55
4	2.57	0.47
5	3.25	0.59
6	2.77	0.50
7	3.57	0.65
8	1.54	0.28
9	3.68	0.67
Reference (Lapatinib)	1.47	0.27
Control (cell free)	5.52	1

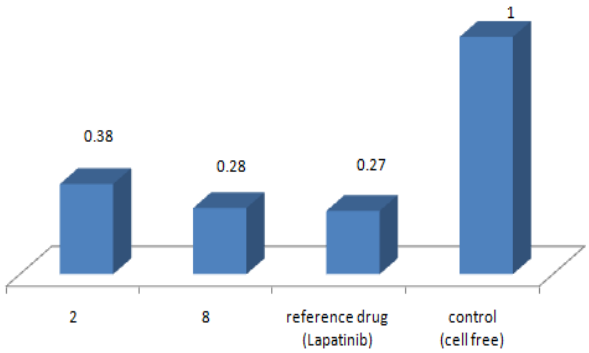


Fig.2most potent active analogs and reference drug

Histopathological investigation

Sections examined showed in Fig.3 & Fig.4 of apoptosis that is characterized by the form of the apoptoticbodies with shrinkage of cell and nucleus (pyknosis) as well as condensation of nuclear chromatin into sharply delineated masses that become marginated against the nuclear membranes. The cells detached from the surrounding tissue and its outlines become convoluted and form extensions.

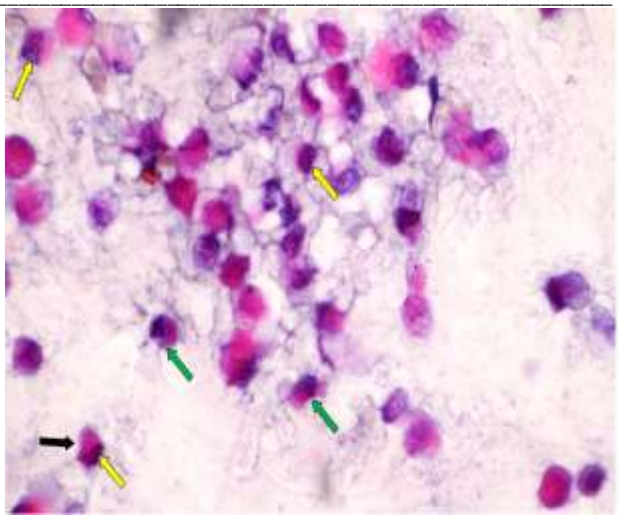


Fig.3 Analog2. photomicrograph showing nuclear and cellular shrinkage (Green arrows), peripheral condensation of chromatin (yellow arrows) and cell membrane irregularities (Black arrow) (H and E original magnification 100X, Oil).

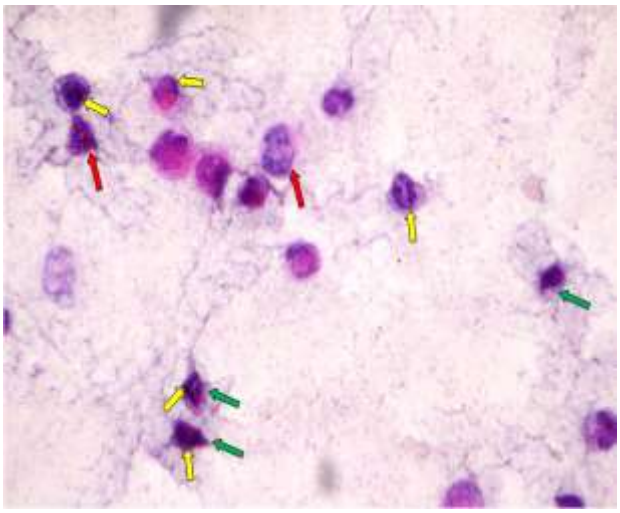


Fig.4 Analog8. photomicrograph showing nuclear shrinkage (Green arrows), peripheral condensation of chromatin (yellow arrows) and nuclear fragmentation (red arrow) (H and E original magnification 100X, Oil).

4. Conclusions

A series of Thalidomide -indole hybrid including compounds 2-9 were designed and synthesized as potential antitumor agents which was characterized by the functionalization aldehyde group with branched active heterocyclic atoms of different linker lengths linked with the styryl group. The antiproliferative activities were evaluated by in vitro against HCT 116 (colon cancer cell line), HepG2

(liver cancer cell line) and MCF-7 (breast cancer cell line). Compound 8and 2 were commonly exhibited the most potent antitumor activity with no toxicity. Moreover, this study shed light on the mechanism of action of these newly synthesized compounds. As obtained from the results, it can be concluded that thalidomide – indole hybrid analogs may exert their anticancer activity. All the Indole-Thalidomide analogs showed good activity as anticancer candidate. In the presented article the mergine of Thiobarbiturate with thalidomide showed the ideal activity against several cancer cell line compared with the other analogs. Further studies on the modification of thalidomide hybrid and use different active cores are still ongoing.

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