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Synthesis and Biological Evaluation of Some *N*-Substituted Quinoxaline Derivatives as Antitumor Agents

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Abstract—A new hybrid molecules containing 1-(*N*-substituted)-quinoxaline derivatives were synthesized from condensation of 3-hydroxy-2-oxo quinoxaline with 2,3-unsaturated carbonyl compounds under different conditions in order to yield ester and amide derivatives. The structure of the prepared compounds was elucidated on the bases of IR, ^{1}H NMR, ^{13}C NMR, mass and elemental analyses. All the prepared compounds were evaluated for their anticancer activity against two cancer cell line (MCF-7 and Hela). Cell cycle analysis of 3-(*p*-methoxyphenyl)-3-(3-hydroxy-2-oxoquinoxalin-1-yl)-2-cyanoacrylic acid hydrazides compound demonstrated cell cycle arrest at S phase and Pre-G1 apoptosis. DNA synthesis inhibitory percentage revealed that this mentioned compound showed equipotent activity to Doxorubicin. Additionally, apoptosis was confirmed by increase the percentage of caspase 3/7 higher than Cisplatin.

Keywords: quinoxaline, anti-proliferative, cell cycle analysis, caspase 3/7, cisplatin

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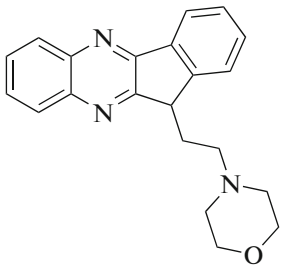
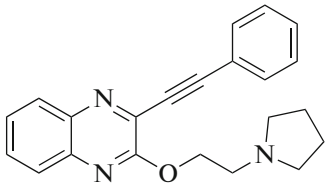
INTRODUCTION

2-Quinolinone derivatives were known to possess a wide range of pharmacological activities [1–7]. For example, dynemicin and streptonigrin are naturally occurring members of the class of antitumor antibiotics [8, 9]. Quinoline derivatives have displayed potent anticancer activity targeting different sites like Topoisomerase, telomerase, farnasyltransferase, tyrosine kinase and protein kinase CK-11. Quinoxaline derivatives are very important class of heterocyclic compounds containing two nitrogen atoms, which is the building block of many useful intermediate in organic synthesis and useful dyes [1]. Quinoxaline derivatives are of great interest to researchers for their significant role in pharmaceutical industry [2, 3]. The importance of these compounds was developed due to their antiviral, kinase inhibitors, anticancer, antibacterial and anti-inflammatory activities [4–8]. Some quinoxaline derivatives such as structures **1**, **2** and **3** are known to biologically active as antiviral, anticancer and antibacterial activities.



Structure **1**



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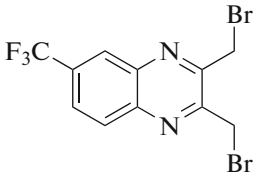

Structure **3**

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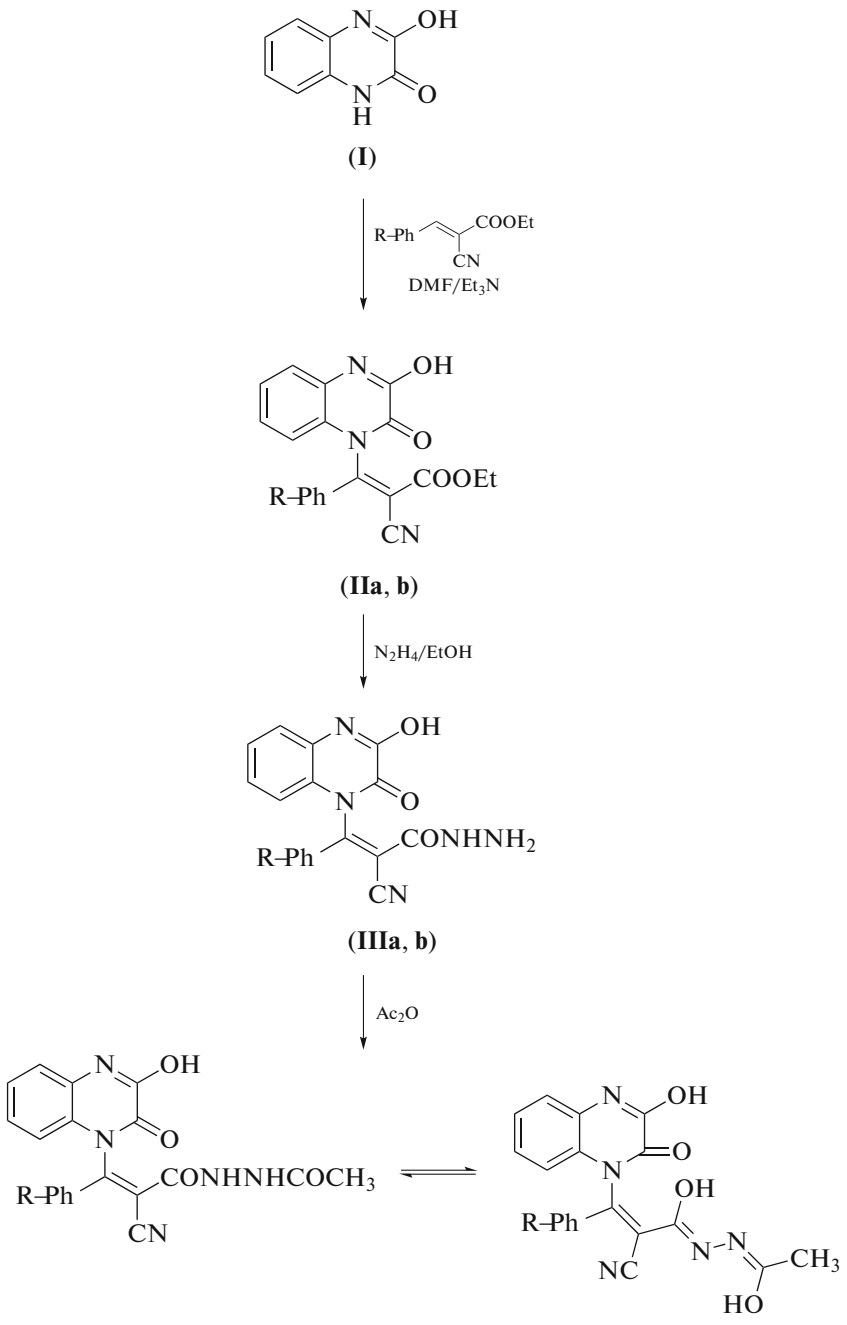


Scheme 1. Synthesis of quinoxaline derivatives **(II)**–**(IV)** **(IIa)**, **(IIIa)** and **(IVa)** R = OCH_3, **(IIb)**, **(IIIb)**, and **(IVb)** R = Cl.

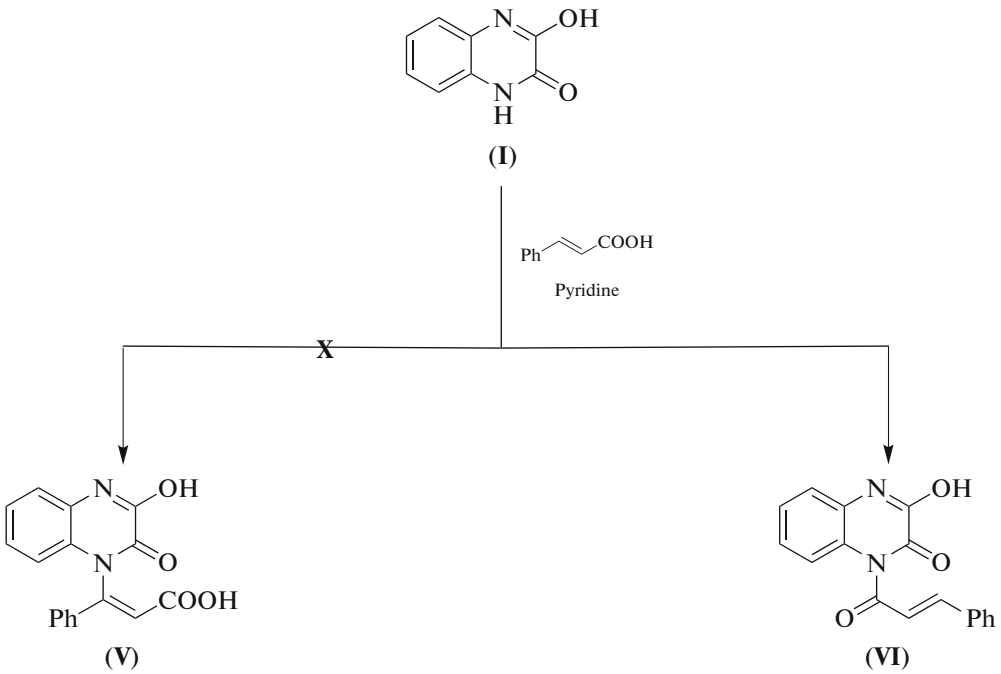
Development of many strategy for the preparation of substituted quinoxaline has been reported [9, 10]. The most common method are the condensation of an 2,3-aryl diamine with 2,3-dicarbonyl compounds in refluxing acetic acid, 4 N hydrochloric acid and/or ethanol. Considering the importance of quinoxaline derivatives, the aim of the present study was to prepare some *N*-substitutedquinoxaline derivatives by using quinoxaline-2,3-dione and examine the cytotoxic activity of the synthesized compounds against two cancer breast cancer cell line and cervix cancer cell line.

RESULTS AND DISCUSSION

Chemistry

The synthetic pathway to obtain the *N*-substituted quinolxaline derivatives are outlined in Schemes 1 and 2. The starting material quinoxaline-2,3-diones **(I)** was

obtained by refluxing 1-phenylenediamine with oxalic acid in 4 N hydrochloric acid according to literature reported [11, 12]. Addition of quinoxaline (**I**) to 2,3-dicarbonyl compounds (namely ethyl 3-(*p*-methoxyphenyl)-2-cynaoacrylate and ethyl 3-(4-chlorophenyl)-2-cynaoacrylate) in dimethyl formamide in the presence of triethylamine as catalyst under reflux led to the formation of ethyl 3-(3-hydroxy-2-oxoquinoxalin-1-yl)-3-aryl-2-cyanoacrylate (**IIa**, **b**). The structure of compounds (**IIa**) was confirmed by its ^{1}H NMR spectrum which showed two signals at δ 1.30 and 4.31 ppm as triplet and quartet for the protons of ethoxy group (CH_3CH_2O–). A two singlet signals for methoxy and hydroxy protons appeared at δ 3.87 and 11.94 ppm confirming the formation of the compound. In addition, the aromatic protons were observed in the region δ 7.08–8.31 ppm as multiplet signals. The ^{13}C-NMR spectrum of compounds (**IIa**) showed two signals at δ 164.02 and δ 162.84 ppm assigned to two carbons of carbonyl groups of quinoxaline and ester. The three signals due to the two carbons at δ 62.56 and 14.50 ppm of ethoxy function and at δ 56.24 ppm of methoxy group. Also, two signals due to the two carbons at δ 155.64 ppm and δ 154.93 ppm attributed to two C–O groups. Aromatic, cyano and olefinic carbons were observed within the expected chemical shift region at δ 134–98.99 ppm.



Scheme 2. Synthesis of quinoxaline derivative (**VI**).

Treatment of ester (**IIa**, **b**) with hydrazine in ethanol afforded the corresponding 3-(aryl)-3-(3-hydroxy-2-oxoquinoxalin-1-yl)-2-cyanoacrylic acid hydrazides (**IIIa**, **b**). The structure of compounds (**IIIa**, **b**) was supported via acetylation of acid hydrazide (**III**) with acetic anhydride under reflux to give *N*-acetyl-3-(aryl)-3-(3-hydroxy-2-oxoquinoxalin-1-yl)-2-cyanoacrylic acid hydrazides (**IVa**, **b**). ^{1}H NMR spectrum of acid hydrazide derivatives (**IVa**) as example showed characteristic five singlet signals at δ 11.49, 11.38, 11.33, 3.85 and 3.74 ppm due to the protons of three hydroxyl (3OH), methoxy (OCH_3) and methyl (CH_3) groups protons of the aromatic ring and NH at δ 6.91–8.64 ppm as multiplet signal were observed. The ^{13}C NMR spectrum of compound (**IVa**) showed two signals at δ 55.75 and δ 41.60 ppm assigned to two carbons of methoxy (OCH_3) and methyl (CH_3) groups.

The carbons of carbonyl and C–O of the compound (**IVa**) appeared in the region at δ 160.93–169.57 ppm, which elucidate the presence of compound (**IVa**) in enol-keto form.

Condensation of 3-hydroxy-2-oxoquinoxaline (**I**) with cinnamic acid in pyridine under reflux was expected to give 3-phenyl-3-(3-hydroxy-2-oxoquinoxalin-1-yl) acrylic acid (**V**), but only 1-(cinnamoyl)-2-oxo-3-hydroxyquinoxaline (**VI**) was afforded. The ^{1}H NMR spectrum of compound (**VI**) showed the protons of hydroxyl group in position 3 of quinoxaline resonated as a broad singlet signal at δ 11.95 ppm. The protons of aromatic rings and olefinic were resonated as multiplet signals in the region at δ 6.52–7.67 ppm. The ^{13}C NMR spectrum of compound (**VI**) showed three signals for the carbons at δ 168.58, 168.40 and δ 155.66 ppm assigned to the two carbonyl (C=O) and C–O groups. In addition, a characteristic carbon sig-

nals in the region at δ 144.00–115.00 ppm for aromatic rings and olefinic carbons.

Evaluation of Biological Activity

In vitro cytotoxic activity against two cancer cell line. The effect of quinoxaline derivatives (**IIa**, **b**), (**IIIa**, **b**), (**IVa**, **b**) and (**VI**) on the viability of two cancer cell lines were studied using MTT assay. The cytotoxicity was assessed using cisplatin as positive control. The two cancer cell line are MCF-7 (breast cancer cell line) and Hela (cervix cancer cell line). Treatment of MCF-7 and Hela cell lines with different concentration of quinoxaline derivatives revealed that some of the tested compounds showed promising cytotoxic activity against MCF-7 cells, and the compounds showed weak cytotoxic activity against Hela cells as concluded from their IC_{50} values as shown in Table 1. Structurally, 3-(aryl)-3-(3-hydroxy-2-oxoquinoxalin-1-yl)-2-cyanoacrylic acid hydrazides (**IIIa**, **b**) showed the highest cytotoxic activity against MCF-7 cells which were more potent than ester precursor (**IIa**, **b**), acetylated derivatives (**IVa**, **b**) and 1-(cinnamoyl)-2-oxo-3-hydroxyquinoxaline (**VI**). Considering compounds (**IIIa**) and (**IIIb**), compound with 4-methoxy phenyl moiety showed increased activity in comparison with compounds containing 4-chloro phenyl suggesting that electron donating group is better than electron withdrawing group. In conclusion, (**IIIa**) and (**IIIb**) were the most potent antitumor compound over MCF-7 cell line, since both of the two compounds possessed the highest cytotoxic activity. The highest cytotoxic compound was (**IIIa**) which had IC_{50} value of 2.61 μM which was comparable of 2.12 μM of cisplatin.

DNA Flow Cytometry Analysis

Cell cycle analysis. To study the mechanism of anticancer activity of compound (**IIIa**), cell cycle analysis was carried out using DNA flow cytometry analysis in MCF-7 cells. MCF-7 cells was incubated with IC_{50} concentration of compound (**IIIa**) and cisplatin for 24 h and then subjected to DNA flow cytometry analysis. As shown in Figs. 1 and 2, exposure of MCF-7 cells to compound (**IIIa**) resulted in interference of the normal cell distribution in the cell cycle profile of MCF-7 cells. Compound (**IIIa**) could enhance the S phase by 20.12% compared with the untreated control. This effect was accompanied by increase in cell percentage in G_1 and G_2/M phase of the cell cycle. These results suggested that compound (**IIIa**) induce cancer cell death *via* S phase arrest with apoptosis inducing activity marked by the presence pre-G_1 peak in the cell cycle distribution profile of MCF-7 cells.

DNA synthesis inhibition assay. In order to investigate the DNA inhibition activity induced by compound (**IIIa**) on MCF-7 cell line. The DNA inhibition percentage (%) of compound (**IIIa**) and cisplatin at their IC_{50} concentration values was investigated using BrdU assay to detect the proliferation of MCF-7 cells for 24 h. As shown in (Fig. 3) compound (**IIIa**) was found to have 81.82% DNA synthesis inhibition percentagein comparison with cisplatin which had 88.05% DNA synthesis inhibition percentage. The results in this experiment confirmed that, the mechanism of compound (**IIIa**) induced MCF-7 cell death was inhibition of DNA synthesis and it is in line withthe previously discussed data.

Activated caspase 3/7 as execution factor for apoptosis. Caspase 3/7 are member of the cysteine aspartic acid-specific protease family and play a key effector roles in apoptosis [13]. The activation of caspase 3/7 was determined in compound (**IIIa**) treated MCF-7 cells at IC_{50} concentration dose value using green flow cytometry assay for 24 h. The result in (Fig. 4) showed an increase in the level of caspase 3/7 percentage by 11.71-fold more than untreated control. Additionally, compound (**IIIa**) revealedcaspase 3/7 activity more than cisplatin as reference compound. In conclusion, compound (**IIIa**) can induce apoptosis in compound (**IIIa**) treated MCF-7 cells and increase caspase 3/7 percentage higher than cisplatin.

Table 1. In vitro antitumor activity ofquinoxaline derivatives **II**, **III**, **IV** and **VI** over MCF-7 and Hela cell lines. Data are expressed as the mean ± three experiments

Compound no.	IC_{50} values, μM	
	MCF-7	Hela
(IIa)	89 ± 2.23	>100
(IIb)	23.41 ± 0.27	63.05 ± 0.97
(IIIa)	2.61 ± 0.32	8.92 ± 0.41
(IIIb)	7.37 ± 0.42	25.05 ± 0.63
(IVa)	6.15 ± 2.21	>100
(IVb)	>100	>100
(VI)	23.34 ± 2.58	19.06 ± 1.91
Cisplatin	2.12 ± 0.09	4.62 ± 0.14

EXPERIMENTAL

Chemistry

Melting point were measured using open capillary tubes on a melt-temp melting point apparatus and are uncorrected. FT-IR spectra were recorded on a Thermofisher Nicolete IS 10 spectrophotometer using KBr pellets. ^1H and ^{13}C NMR (400 MHz) spectra recorded on a Brucker Avance-400 spectrometer using DMSO-d_6 as solvent- chemical shifts are reported on ppm downfield from internal tetramethylsilane and are given in the scale. The elemental analysis was carried out on a Perkin-Elmer 2400 series II CHN analyzer. All synthesized quinoxaline derivatives give satisfac-

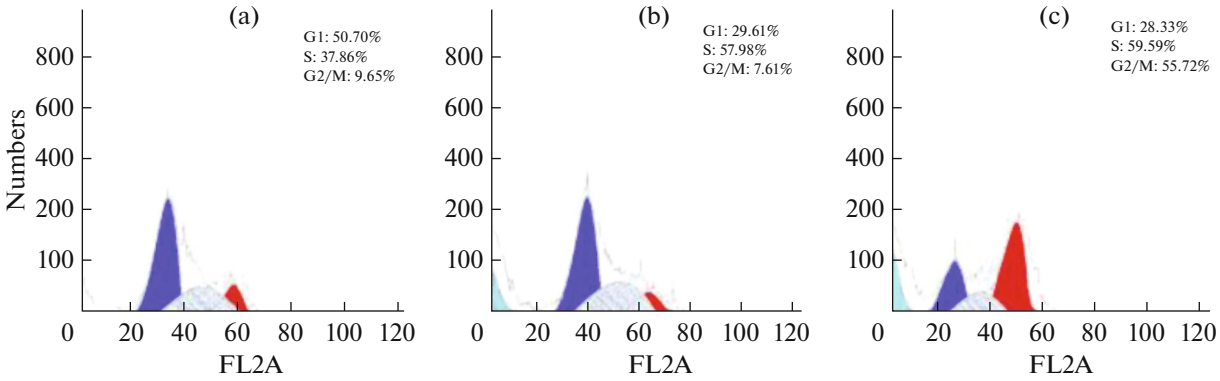


Fig. 1. Effect of compound (**IIIa**) in MCF-7 cell distribution after treatment for 24 h. (a) Control group, (b) compound (**IIIa**) and (c) cisplatin.

tory elemental analysis within 0.4% of the theoretical values. Chemicals and solvents were purchased from commercial sources an analytical grade purity.

Ethyl 3-aryl-3-(3-hydroxy-2-oxoquinoxalin-1-yl-2-cyanoacrylate (IIa, b). A mixture of equimolar quantity of 3-hydroxy-2-oxoquinoxaline (**I**) (0.01 mol) and 2,3-unsaturated ester (0.01 mol) in dimethyl formamide in the presence of triethyl amine (3 mL) was heated under reflux for 4 h. The reaction mixture was cooled and poured into water and neutralized with dilute hydrochloric acid (2%). The solid product was filtered off, washed with water, dried and crystallized from ethanol to give (**II**).

Ethyl 3-(4-methoxyphenyl)-3-(3-hydroxy-2-oxoquinoxalin-1-yl)-2-cyanoacrylate (IIa). Pale green crystals, yield 76%, mp 75–77°C. IR (KBr): 3415 (OH), 2214 (CN), 1716 (C=O), 1605, 1585 (C=C), 1182, 1124, 1015 (C–O) cm^{-1}. ^1H NMR (DMSO-d_6) δ: 1.30 (t, 3H, CH_3), 3.87 (s, 3H, OCH_3), 4.31 (q, 2H, OCH_2), 7.08–8.31 (m, 8H, Ar-H), 11.94 (s, 1H, OH) ppm. ^{13}C NMR (DMSO-d_6) δ: 164.02, 162.84 (C=O), 155.64, 154.85 (C–O), 134.00, 126.05, 124.42, 123.48, 116.74, 115.58, 115.44, 98.99 (C aromatic and CN), 62.56 (OCH_2), 56.24 (OCH_3), 14.50 (CH_3) ppm. MS: m/z (%) = 391 (M^+, 16.30). Anal. calcd. for $C_{21}H_{17}N_3O_5$ (391): C, 64.45; H, 4.35; N, 10.74. Found: C, 64.24; H, 4.13; N, 10.47.

Ethyl 3-(4-chlorophenyl)-3-(3-hydroxy-2-oxoquinoxalin-1-yl)-2-cyanoacrylate (IIb). Yellow crystals, yield 77%, mp 82–84°C. IR (KBr): 3421 (OH), 2224 (CN), 1718 (C=O), 1608, 1595 (C=C), 1171, 1118, 1017 (C–O) cm^{-1}. ^1H NMR (DMSO-d_6) δ: 1.31 (t, 3H, CH_3), 4.21 (q, 2H, OCH_2), 7.21–8.45 (m, 8H, Ar-H), 11.92 (br. s, 1H, OH) ppm. MS: m/z (%) = 397 (M^+ + 2, 3.30), 395 (M^+, 10.30). Anal. calcd. for $C_{20}H_{14}N_3ClO_4$ (395): C, 60.76; H, 3.54; N, 10.63. Found: C, 60.46; H, 3.33; N, 10.41.

3-(Aryl)-3-(3-hydroxy-2-oxoquinoxalin-1-yl)-2-cyanoacrylic acid hydrazides (IIIa, b). A mixture of ester derivatives (**II**) (0.10 mol) and hydrazine hydrate (0.03 mol) in ethanol (70 mL) was heated under reflux for 4 h. The reaction mixture was cooled, then poured into water and neutralized with dilute hydrochloric acid (2%). The solid formed was filtered off, washed with water, dried and the product was crystallized from ethanol to give (**III**).

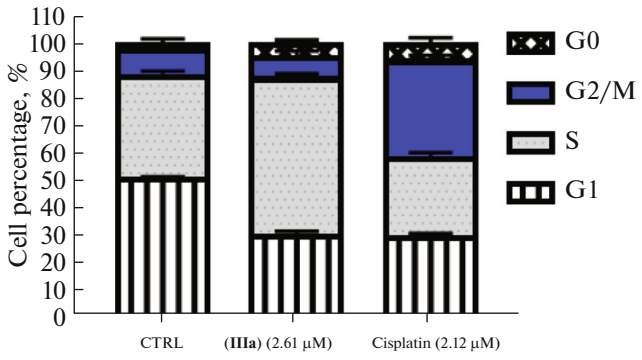


Fig. 2. Graphical presentation of the effect of compound (**IIIa**) in cell distribution after treatment for 24 h compared with cisplatin.

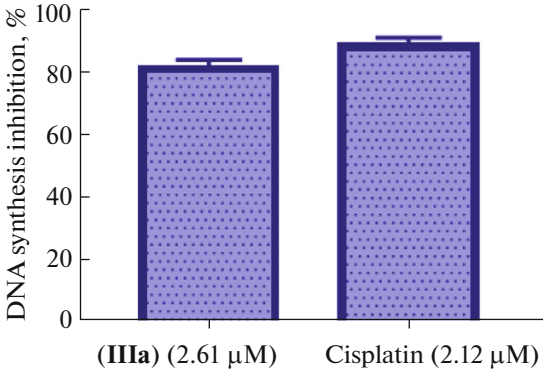


Fig. 3. DNA synthesis inhibition percentage after treatment with (**IIIa**) and cisplatin at their IC_{50} (μM) for 24 h.

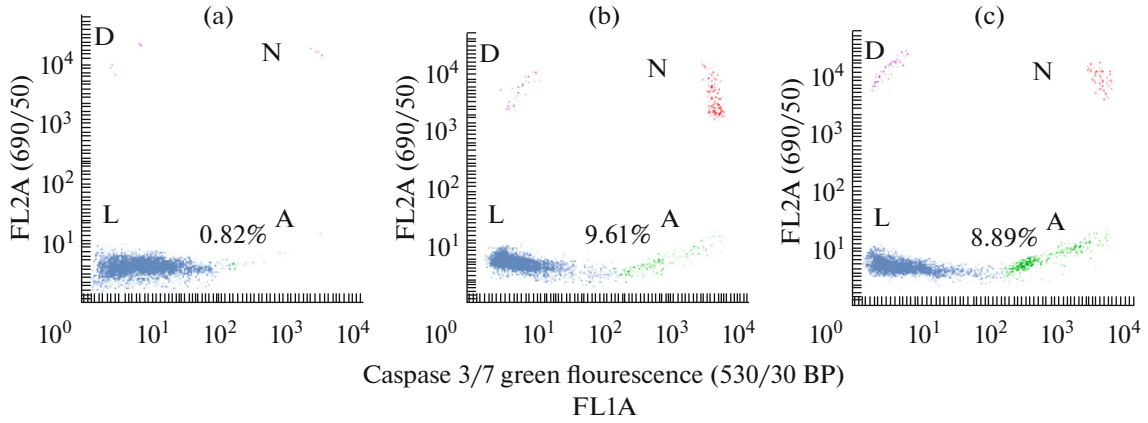


Fig. 4. Green flow cytomertic assay of caspase 3/7 (%) of the tested compounds (**IIIa**) compared to cisplatin at their IC_{50} (μM). (a) Control group, (b) compound (**IIIa**) and (c) cisplatin.

3-(4-Methoxyphenyl)-3-(3-hydroxy-2-oxoquinoxalin-1-yl)-2-cyanoacrylic acid hydrazides (IIIa). Colorless crystals, yield 68%, mp 135–137°C. IR (KBr): 3408 (OH), 3218, 3152 (NH_2, NH), 2320 (CN), 1693 (C=O), 1605, 1580 (C=C), 1062 (C–O) cm^{-1}. ^1H NMR (DMSO-d_6) δ: 3.83 (s, 3H, OCH_3), 4.19 (s, 2H, NH_2), 6.92–7.95 (m, 8H, Ar-H), 8.64 (s, 1H, CONH), 11.93 (s, 1H, OH) ppm. ^{13}C NMR (DMSO-d_6) δ: 162.14, 161.02 (C=O), 155.86, 154.65 (C–O), 130.46, 129.11, 127.97, 127.01, 126.06, 123.49, 115.58, 114.87, 114.74, 114.63, 114.51 (C aromatic and CN), 55.85 (OCH_3) ppm. MS: *m/z* (%) = 377 (M^+, 15.20). Anal. calcd. for $C_{19}H_{15}N_5O_4$ (377): C, 60.48; H, 3.98; N, 18.57. Found: C, 60.40; H, 3.63; N, 18.33.

3-(4-Chlorophenyl)-3-(3-hydroxy-2-oxoquinoxalin-1-yl)-2-cyanoacrylic acid hydrazides (IIIb). Pale yellow crystals, yield 63%, mp 127–129°C. IR (KBr): 3445 (OH), 3320, 3215 (NH_2, NH), 2221 (CN), 1696 (C=O), 1605, 1585 (C=C), 1019 (C–O) cm^{-1}. ^1H NMR (DMSO-d_6) δ: 4.23 (s, 2H, NH_2), 6.95–8.23 (m, 8H, Ar-H), 8.81 (s, 1H, CONH), 11.96 (br. s, 1H, OH) ppm. MS: *m/z* (%) = 383 (M^+ + 2, 2.30), 381 (M^+, 7.20). Anal. calcd. for $C_{18}H_{12}N_5ClO_3$ (381): C, 56.63; H, 3.17; N, 18.34. Found: C, 56.36; H, 3.03; N, 18.52.

***N*-Acetyl 3-(aryl)-3-(3-hydroxy-2-oxoquinoxalin-1-yl)-2-cyanoacrylic acid hydrazides (IVa, b).** A solution of acrylic acid hydrazide derivatives (**III**) (0.01 mol) in acetic acid anhydride (25 mL) was heated under reflux for 2 h, then cooled and poured into ice-water. The reaction mixture was left 24 h and the solid formed was filtered off, washed with water and dried. Finally, the product was crystallized from ethanol to give compound (**IV**).

***N*-Acetyl 3-(4-methoxyphenyl)-3-(3-hydroxy-2-oxoquinoxalin-1-yl)-2-cyano acrylic acid hydrazides (IVa).** Orange crystals, yield 67%, mp 221–223°C. IR (KBr): 3425 (OH), 3225 (NH), 2225 (CN), 1698–1693 (br, C=O), 1605, 1595 (C=C), 1128, 1087 (C–O) cm^{-1}. ^1H NMR (DMSO-d_6) δ: 3.59 (s, 3H, CH_3), 3.83 (s, 3H, OCH_3), 3.39 (s, 2H, NH_2), 6.86–8.17 (m, 8H, Ar-H), 8.64 (s, 2H, 2CONH), 11.33 (s, 1H, OH) , 11.38 (s, 1H, OH), 11.49 (s, 1H, OH) ppm.^{13}C NMR (DMSO-d_6) δ: 169.57, 169.14, 163.44 (C=O), 162.14, 161.32, 160.93 (C–O), 147.14, 143.20, 142.87 (C=N), 130.45, 129.18, 128.88, 128.79, 127.39, 127.31, 127.24, 127.16, 127.05, 114.86, 114.76, 114.61 (C aromatic, olefinic and CN), 55.75 (OCH_3) ppm. MS: *m/z* (%) = 419 (M^+, 3.20). Anal. calcd. for $C_{21}H_{17}N_5O_5$ (419): C, 60.14; H, 4.06; N, 16.71. Found: C, 59.98; H, 3.83; N, 16.43.

***N*-Acetyl 3-(4-chlorophenyl)-3-(3-hydroxy-2-oxoquinoxalin-1-yl)-2-cyanoacrylic acid hydrazides (IVb).** Orange crystals, yield 63%, mp 205–2077°C. IR (KBr): 3430 (OH), 3227 (NH), 2223 (CN), 1699–1693 (br, C=O), 1607, 1585 (C=C), 1078, 1023 (C–O) cm^{-1}. ^1H NMR (DMSO-d_6) δ: 3.43 (s, 3H, CH_3), 4.23 (s, 2H, NH_2), 7.12–8.24 (m, 8H, Ar-H), 8.71 (s, 1H, CONH), 11.34 (s, 1H, OH), 11.39 (s, 1H, OH), 11.78 (s, 1H, OH) ppm. MS: *m/z* (%) = 425 (M^+ + 2, 2.57), 423 (M^+, 9.63). Anal. calcd. for $C_{20}H_{14}N_5ClO_4$ (423): C, 56.68; H, 3.33; N, 16.52. Found: C, 56.45; H, 3.08; N, 16.63.

1-(Cinnamoyl)-2-oxo-3-hydroxyquinoxaline (VI). A mixture of 3-hydroxy-2-oxoquinoxaline (**I**) (0.01 mol) and cinnamic acid (0.01 mol) in pyridine (30 mL) was heated under reflux for 6 h, then cooled and poured into water. The reaction mixture was neutralized with dilute hydrochloric acid (2%) and the resulting solid was filtered off, washed with water and dried. Finally, the product was crystallized from butanol to give compound (**VI**) as colorless crystals.

Yield 73%, m.p. 197–199°C. IR (KBr): 3446 (br, OH), 1682 (C=O), 1630 (C=N), 1605, 1583 (C=C), 1230 (C–O) cm^{-1}. ^1H NMR (DMSO-d_6) δ: 6.52–7.67 (m, 11H, Ar-H and olefinic H), 11.95 (br. s, 1H, OH)

ppm. ^{13}C NMR (DMSO-d_6) δ: 168.58, 168.40 (C=O), 155.66 (C–O), 143.89, 143.68, 134.88, 130.55, 129.35, 128.58, 126.06, 123.48, 120.65, 120.36, 115.59 (C aromatic and olefinic) ppm. MS: m/z (%) = 292 (M^+, 32.35). Anal. calcd. for $C_{17}H_{12}N_2O_3$ (292): C, 69.86; H, 4.11; N, 9.59. Found: C, 69.59; H, 3.89; N, 9.35.

Biological Evaluation

Anti-tumor activity against two cancer cell line (MCF-7 and Hela). The cytotoxic activity was measured in vitro for the newly synthesized compounds using the MTT assay. Cells were plated in 96-multiwall plate (10^5 cells/well) for 24 h before treatment with the compounds. Test compounds were dissolved in dimethyl sulfoxide. Different concentrations of the compound under test (10, 25, 50, and 100 μM) were added to the cell's monolayer. Triplicate wells were prepared for each individual concentration. Monolayer cells were incubated with the compound(s) for 48 h at 37°C and in atmosphere of 5% CO_2. After 48 h, cells were fixed, washed and stained with 40 μL of MTT solution (5 mg/mL of MTT in 0.9% NaCl) in each well was added and incubated for an additional 4 h. MTT crystals were solubilized by adding 180 μL of acidified isopropanol/well and the plate was shaken at room temperature, followed by photometric determination of the absorbance at 570 nm using ELISA reader. The molar concentration required to inhibit 50% of cell viability (IC_{50}) was calculated and compared with the reference drug doxorubicin. The surviving fractions were expressed as means ± S.E.M.

Cell cycle analysis of compound (IIIa). MCF-7 cells, (3.0×10^5 cells/well) and incubated at 37°C for 12 h. The target cells were then treated with the compound (**IIIa**) at its IC_{50} concentration for 24 h. After treatment, cells were collected and fixed with 75% ethanol at 20°C overnight, then, cells were washed with PBS followed by centrifugation and incubated with (10 mg/mL) RNase (Sigma, USA) and (5 mg/mL) propidium iodide (PI, Sigma) before flow cytometry analysis (FACSCalibur cytometer using Cellquest software, BD Bioscience, USA).

DNA synthesis inhibition assay. MCF-7 cells (2×10^4/well) were treated with compounds (**IIIa**) and Cisplatinat their IC_{50} values for 24 h. DNA inhibition activity were determined using BrdU Kit according to the manufacturer's instruction. Briefly, cytotoxicity test were performed using the cell proliferation ELISA. After that cells were treated with BrdU solution for 2 h at 37°C, then treated with specific antibody and incubated with further 90 min. Then, the cells were washed with PBS until the color development was adequate. Optical density of the samples were measured in an ELISA reader. Finally, the percentage of cell growth was measured versus positive control.

Caspase 3/7 Assay of Compound (IIIa)

The enzymatic activities of caspase 3/7 were assayed in cell lysates (100 mg protein in 50 mL lysis buffer) using caspase 3/7 green flow cytometry assay kit as per the manufacturer's instructions. Briefly, control and compound (**IIIa**) treated MCF-7 cells (2.5×10^5/mL) were washed with ice cold PBS, cell lysates. were prepared and subsequently protein concentration was estimated. Lysates were combined with reaction buffer and incubated with specific colorimetric substrates (Caspase 3/7 Detection Reagent) at 37°C for 6 h. The samples were measured at 488 nm in BD FACS Calibur flow cytometer. All experiments were done in triplicates.

CONCLUSION

In summary, hybrid molecules having 1-(*N*-substituted) quinoxaline derivatives (**II**–**VI**) were prepared from 3-hydroxy-2-oxo quinoxaline (**I**) with 2,3-unsaturated carbonyl compounds and cinnamic acid under different conditions. The structure of the prepared compound were confirmed via IR, ^{1}H NMR, ^{13}C NMR, MS and elemental analyses. All the prepared compounds were evaluated for their anticancer activity against two cancer cell line (MCF-7 and Hela). The most promising compound in this series was 3-(4-methoxyphenyl)-3-(3-hydroxy-2-oxoquinoxalin-1-yl)-2-cyanoacrylicacid hydrazides (**IIIa**) which significantly inhibited MCF-7 cells with IC_{50} value of 2.61 μM. Moreover cell cycle analysis of compound (**IIIa**) demonstrated cell cycle arrest at S phase and Pre-G1 apoptosis. DNA synthesis inhibitory percentage revealed that compound (**IIIa**) showed equipotent activity to doxorubicin. Additionally, Apoptosis was confirmed by increase the percentage of caspase 3/7 higher than cisplatin.

COMPLIANCE WITH ETHICAL STANDARDS

This article does not contain any studies involvinghuman participants performed by any of the authors anddoes not contain any studies involving animals performedby any of the authors.

Conflict of Interests

The authors report no conflicts of interest.

SUPPLEMENTARY MATERIALS

Supplementary materials are available for this article at https://doi.org/10.1134/S1068162020030097 and are accessible for authorized users.

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