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Research Article

Investigation of the Cytotoxic Potencies of substituted dipyrrolo[1,2-a:2',1'-c]pyrazines and substituted pyrrolo[2'',1'':3',4']pyrazino[1',2':1,5]pyrrolo[2,3-*d*]pyridazine-8(9*H*)-ones

Azam Barghi Lish[a], Eskandar Kolvari[a*], Alireza Foroumadi[b,c*], Nadiya Koukabi[a]

[a]*Department of Chemistry, Semnan University, Semnan, Iran*

[b]*Department of Medicinal Chemistry, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran*

[c]*Drug Design and Development Research Center, The Institute of Pharmaceutical Sciences (TIPS), Tehran University of Medical Sciences, Tehran, Iran*

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ABSTRACT

Cancer is the world's second leading cause of death after cardiovascular diseases. The data reported by the World Health Organization (WHO) about this disease indicates a troubling increase in both prevalence and mortality over the past decade. Consequently, significant efforts have been directed toward the discovery and development of new and potent anticancer agents. In an attempt to find and develop further new compounds possessing cytotoxic potencies, the efficient, simple, and multi-step synthetic routes were employed to afford various substituted dihydrodipyrrolo[1,2-a:2',1'-*c*]pyrazine-2,3-dicarboxylates **8a-8s**, which were subsequently subjected to cyclization in the presence of hydrazine hydrate to produce tetrahydropyrrolo[2",1":3',4']pyrazino[1',2':1,5]pyrrolo[2,3-*d*]pyridazine-8(9H)-ones **10a-10q**. Afterwards, their cytotoxicity were examined against five human cancerous cell lines, including MCF7, HeLa, SW480, HepG2, and A549 by using the MTT colorimetric assay. Considering the results, further evaluations were conducted on the compound **8l**, which exhibited the induction of apoptosis and G0 cell cycle arrest in MCF7 and A549 cells.

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[*] **.Corresponding author 1:** Assistant Professor of organic Chemistry. *E-mail address: kolvari@semnan.ac.ir*
[*] **.Corresponding author 2:** Professor of Medicinal Chemistry. *E-mail address: aforoumadi@yahoo.com*

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1. Introduction

Cancer emerges as a complicated disease caused by an intricate interaction of external and internal factors. It ranks as the second leading cause of mortality globally, following closely behind cardiovascular diseases. Data from the World Health Organization (WHO) in 2020 underscored that the global mortality caused by cancer continued to be a significant concern, with an estimated increase in deaths compared to previous decades. Projections for 2045 suggest a continued rise in cancer-related mortality due to factors such as aging populations, lifestyle changes, and environmental influences [1]. Among various clinical strategies and medical technologies, including surgery, radiotherapy, and chemotherapy, the latter has been used more frequently for the cancer treatment. Consequently, there is a crucial need for the discovery and development of effective chemotherapeutic agents that not only demonstrate efficacy but also exhibit reduced side effects and toxicity profiles [2-4]. Up to now, numerous chemotherapeutic agents have been extensively designed, synthesized, and developed to address diverse cancer types through inhibition of various pathways, including kinase [5, 6], tubulin polymerization [7, 8], topoisomerase [9, 10], BET bromodomain [11-13], hedgehog signaling [14, 15], heat shock protein 90 (HSP90) [16, 17], centromere-associated protein E (CENP-E) [18, 19], phosphoribosyltransferase (Nampt) [20, 21], and translocator protein (TSPO) [22, 23].

Despite the effectiveness of these heterocyclic anticancer agents, their undesirable side effects, toxicity levels, and development of drug resistance have encouraged researchers to identify compounds with reduced adverse effects [24-26]. One of the pivotal strategies used by researchers in the field of medicinal chemistry is hybridization, a synthetic method increasingly utilized in the drug discovery and development process. Hybridization involves combining two or more pharmacophores to synthesize additional bioactive small molecules. This approach is designed not only to address the shortcomings of individual compounds but also to enhance their potency and selectivity [27].

Numerous substituted pyrroles [28-30], pyrazines [31, 32], and pyridazines [33, 34] have already been identified as potential anti-cancer agents. Our recent advancement in hybridization strategy of these valuable pharmacophores to afford various substituted dihydrodipyrrolo[1,2-a:2',1'-*c*]pyrazine-2,3-dicarboxylate derivatives **8a-8s** and tetrahydropyrrolo[2",1":3',4']pyrazino[1',2':1,5]pyrrolo[2,3-*d*]pyridazine-8(9H)-one derivatives **10a-10q** as potent cytotoxic compounds [35] led us to extend this study and conduct further evaluations on these compounds. Therefore, they were synthesized once more to investigate their cytotoxic potencies against five human cancerous cell lines, named MCF7 (breast cancer), HeLa (cervical cancer), SW480 (colorectal adenocarcinoma), HepG2 (hepatocellular carcinoma), and A549 (lung carcinoma), in comparison to Sorafenib (used as a reference drug). Additionally, their apoptosis-inducing activity and cell-cycle arrest were examined.

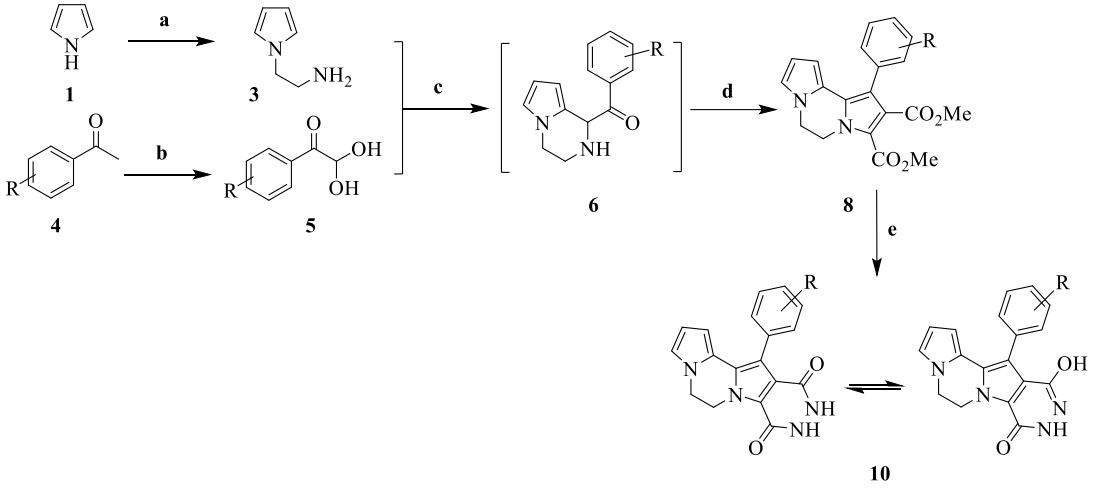
2. Results and discussion

2-1. Chemistry

The multi-step synthetic route to achieve the targeted dihydrodipyrrolo[1,2-a:2',1'-c]pyrazine-2,3-dicarboxylate derivatives **8a-8s** and tetrahydropyrrolo[2",1":3',4']pyrazino[1',2':1,5]pyrrolo[2,3-d]pyridazine-8(9H)-one derivatives **10a-10q** is outlined in the Scheme 1. Initially, *N*-aminoethylpyrrole **3** was synthesized through the nucleophilic substitution reaction between 1*H*-pyrrole and 2-chloroethan-1-amine hydrochloride **2**. On the other hand, a series of substituted arylglyoxal derivatives **5** were obtained from the corresponding substituted acetophenones **4** according to the

previously reported procedure. Subsequently, compounds **3** and **5** went through the condensation reaction in the presence of acetic acid (HOAc) in dry dichloromethane (DCM) at ambient temperature to give adducts **6**, which had two functional groups, NH-acid and carbonyl group, in appropriate position. Therefore, they could be applied for further one-pot Wittig reaction.

Aftercompletion of the preparation reaction of compounds **6** according to the TLC analysis, triphenylphosphine was added to the reaction container, and the mixture was added dropwise to a stirring solution of dimethyl acetylenedicarboxylate (DMAD) in DCM over 10 min. The resulted mixture was continued stirring at room temperature for an additional hour, yielding desirable dihydrodipyrrolo[1,2-a:2',1'-c]pyrazine-2,3-dicarboxylate derivatives **8**. Finally, compounds **8** went through a cyclization reaction with hydrazine **9** under the reflux conditions in ethanol (EtOH), resulting in the preparation of the desired target compounds **10**.



Scheme 1. Reagents and conditions: (a) 2-chloroethan-1-amine hydrochloride **2**, K_2CO_3, DMF, 80 °C, 12h; (b) dioxane, water, 50 °C, 4h; (c) HOAc (20 mol%), DCM, r.t., 1h; (d) PPh_3, DMAD **7**, DCM, r.t., 1h; (d) hydrazine **9**, EtOH, reflux, 10h.

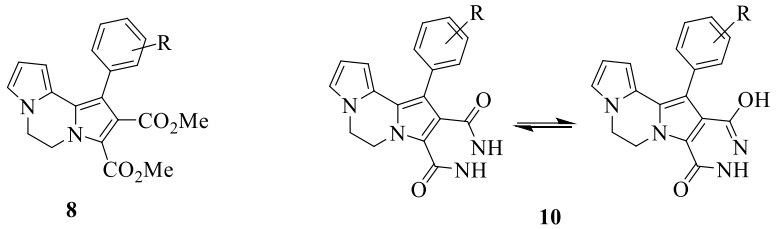
After optimization of the reaction conditions, with the purpose of extending this reaction, various substituted acetophenones bearing electron-donating like alkyl, hydroxy, or methoxy groups, as well as electron-withdrawing, like chlorine, bromine, or nitro groups were used in the presented synthetic routes to afford a large library of targeted compounds 8a-8s and 10a-10q. Finally, the structures of the isolated products were deduced on the basis of their 1H, and 13C NMR spectroscopy, and mass spectrometry. Partial assignments of these resonances are given in the Experimental Part.

2-2. In vitro anti-proliferative activity

The targeted compounds 8a-8s and 10a-10q were evaluated for their in vitro antitumor activities against five human cancerous cell lines, including MCF7, HeLa, SW480, HepG2, and A549 by using the MTT colorimetric assay to investigate the role of different parts of skeleton. In this study, Sorafenib, a well-known chemotherapeutic drug, was utilized as a positive control. The results are depicted in Table 1. The cytotoxicity is expressed as the concentration that inhibit 50% of cell viability (IC50). As illustrated in Table 1, in general, the target compounds exhibited good cytotoxicity activity. Across the first series, compounds 8l, bearing chlorine atom at C-4 positions as well as compounds 8m and 8n, bearing bromine atom C-3 and C-4 positions, exhibited the most potent anti-proliferative activities. Particularly, they emerged several times more potent than the standard drug

against MCF7 cell line. Within the second series, compound 10i, bearing 3-Cl, emerged as the most potent compounds. Therefore, these compounds were selected for further evaluations, named apoptosis-inducing activity. Moreover, given their superior anti-proliferative activities against MCF-7 and A549 cells, these cell lines were selected for subsequent investigation.

Table 1: *In vitro* anti-proliferative activities of compounds **8a-8s** and **10a-10q** against five cancer cell lines [a]



8 **10**

Compound	R	MCF7	HeLa	SW480	HEPG-2	A549
8a	H	23.34±0.1	34.44±1.6	24.52±0.7	7.34±0.01	19.06±0.1
8b	2-OH	8.22±0.09	46.77±1.4	67.65±2.5	48.10±0.2	18.89±0.09
8c	3-OH	42.87±0.3	32.21±0.3	70.15±2.7	24.57±0.3	26.12±0.15
8d	2-Me	51.92±0.4	43.96±1.5	45.07±1.7	43.92±0.1	44.15±0.22
8e	3-Me	13.02±0.1	42.83±1.8	65.60±1.7	9.84±0.06	27.48±0.07
8f	4-Me	45.04±0.4	41.43±0.4	61.29±2.0	30.69±0.4	47.98±0.2
8g	3-OMe	62.38±2.9	41.60±1.1	56.53±1.2	46.96±0.3	27.29±0.1
8h	4-OMe	8.52±0.02	70.15±1.9	64.16±2.2	53.71±0.5	7.44±0.06
8i	2-F	50.82±0.5	41.76±1.2	69.58±1.8	3.33±0.07	24.63±0.22
8j	4-F	68.94±0.6	73.77±2.9	46.79±1.1	62.99±0.6	67.10±1.8
8k	3-Cl	24.66±0.2	23.15±0.2	63.47±1.3	28.46±0.2	37.56±0.31
8l	4-Cl	2.80±0.03	6.11±0.08	14.98±0.3	9.94±0.07	2.53±0.05
8m	3-Br	3.35±0.01	11.79±0.1	61.23±0.4	27.76±0.1	1.15±0.03
8n	4-Br	2.67±0.2	11.35±0.09	6.84±0.05	7.20±0.035	2.09±0.08
8o	3-NO_2	14.63±0.07	33.65±1.4	65.80±0.2	69.32±0.5	20.58±0.5
8p	4-NO_2	3.72±0.55	43.49±1.6	29.40±1.0	67.40±0.3	2.86±0.05
8q	3-OMe-4-OH	9.17±0.04	4.50±0.03	21.79±0.7	74.26±2.1	48.24±0.6
8r	2,4-Cl_2	5.69±0.03	18.98±0.1	66.34±1.2	67.49±1.3	10.13±0.05
8s	3,4,5-$(OMe)_3$	4.69±0.09	8.94±0.03	30.50±0.1	26.26±0.24	6.87±0.03
10a	H	19.87±0.38	42.06±0.08	15.40±0.08	4.96±0.13	17.92±0.07
10b	3-OH	10.18±0.12	13.07±0.09	34.87±0.14	10.31±0.28	31.07±0.26
10c	2-Me	26.35±0.20	3.69±0.38	31.19±0.24	4.14±0.36	8.30±0.03
10d	3-Me	18.86±0.47	2.48±0.20	23.37±0.14	13.95±0.05	3.43±0.18
10e	4-Me	36.94±0.28	25.47±0.20	12.83±0.24	29.34±0.06	32.23±0.18
10f	3-OMe	10.50±0.36	8.72±0.16	14.83±0.22	10.99±0.46	7.43±0.27
10g	4-OMe	23.61±0.26	23.61±0.26	19.62±0.38	11.24±0.06	9.54±0.33
10h	4-F	9.32±0.38	9.32±0.38	26.35±0.09	3.69±0.12	8.31±0.16
10i	3-Cl	7.38±0.24	7.38±0.24	14.40±0.28	10.51±0.18	5.21±0.12
10j	4-Cl	16.42±.03	16.42±0.03	22.09±0.12	15.76±0.33	18.89±0.27
10k	3-Br	7.90±0.36	7.90±0.36	83.37±0.17	38.25±0.12	7.90±0.28
10l	4-Br	12.68±0.05	3.02±1.1	14.58±0.3	84.95±3.4	67.00±2.0
10m	3-NO_2	8.10±0.02	4.96±0.1	60.66±1.1	27.77±1.8	2.08±0.01
10n	4-NO_2	10.37±0.03	55.30±1.8	67.70±1.5	26.37±0.9	68.37±1.4
10o	3-OMe-4-OH	6.80±0.05	9.42±0.6	71.87±1.1	38.45±0.4	6.23±0.03
10p	2,4-Cl_2	9.36±0.06	32.93±0.6	60.55±0.4	44.61±0.5	9.15±0.02
10q	3,4,5-$(OMe)_3$	20.48±0.1	4.37±0.06	45.69±0.06	71.60±3.2	57.07±0.6
Sorafenib	-	**7.06±0.1**	**5.45±0.1**	**19.8±0.1**	**29.6±0.3**	**2.81±0.7**

[a] Values are the mean ± SD. All experiments were performed at least three times.

2-3. Apoptosis-inducing activity

Programmed cell death, known as apoptosis, plays a crucial role in various biological functions. Consequently, disruptions in this process are implicated in diseases like cancer. The effectiveness of compounds 8n, 8m, 8l, and 10i in inducing apoptosis at IC50 concentration was assessed on MCF-7 and A549 cell lines. Flow cytometry analysis using propidium iodide (PI) and annexin V-fluorescein isothiocyanate (annexin V-FITC) was employed for this experiment. DMSO and sorafenib

were used as the negative control and positive control, respectively. Compound 10i was unable to induce apoptosis and remained live in both cell lines; while other compounds were able to induce apoptosis.

The proportions of necrotic, apoptotic, and viable cells for each of them are illustrated in Fig. 1. A single experimental trial was conducted with one replication. The X-axis represents propidium iodide (PI), while the Y-axis represents Annexin V. Quadrant 1 (Q1) denotes necrotic cells, Quadrant 2 (Q2) represents late apoptotic cells, Quadrant 3 (Q3) signifies early apoptotic cells, and Quadrant 4 (Q4) indicates live cells. As depicted in Fig. 2, compound 8l emerged as a the most potential compound which could induce cell death in the apoptotic pathway. Moreover, this derivative exhibited a higher percentage of total apoptosis in A549 cells in comparison with MCF-7 cells. This data led us to proceed our investigation on this compound.

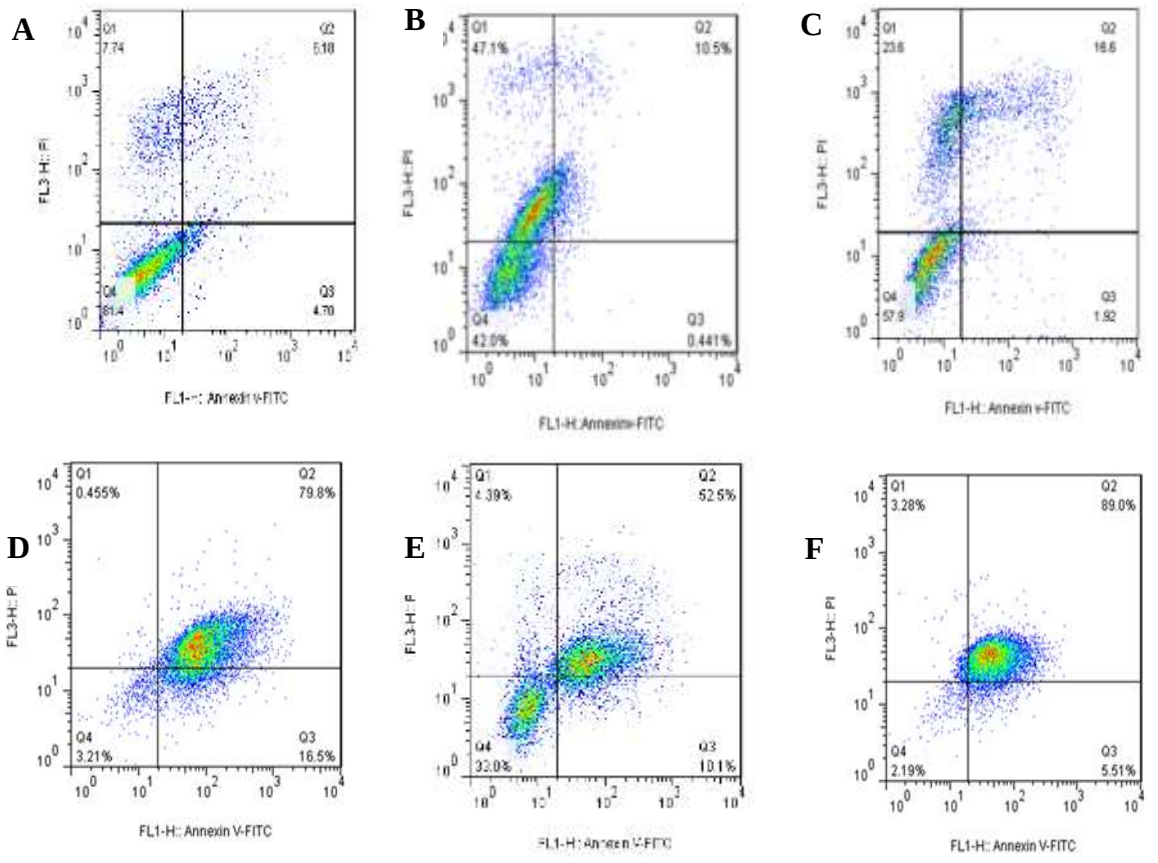


Fig. 1. Apoptosis analysis of MCF-7 and A549 cells induced by **8l**, **8m**, and **8n**: (A) DMSO; (B, C) sorafenib; (D) **8l**; (E) **8m**; (F) **8n** against MCF-7.

2-4. Cell cycle arrest

To find further information about the anti-tumor mechanisms of the compound 8l, its effects on cell cycle progression in MCF-7 and A549 cells at their IC50 concentrations was investigated using Annexin V-FITC/PI dual staining assay. The percentage of G0 cell increased to 54.3% and 65.5% in MCF-7 and A549 cell lines, respectively. While, these figures were 2.02 and 2.25% for non-treated cells and sorafenib in this phase (Fig. 2).

Therefore, compound 8l could be resulted to arrest cell growth in G0 phase and prevent tumor growth by stopping cancer cells from dividing and proliferating. It must be noted that arresting cells in the G0 phase can trigger apoptotic pathways in cancer cells to eliminate damaged or unwanted cells.

Moreover, compounds arresting cells in the G0 phase are sensitive to other cancer treatments, including chemotherapy or radiation therapy, resulting to their effectiveness enhancement [36].

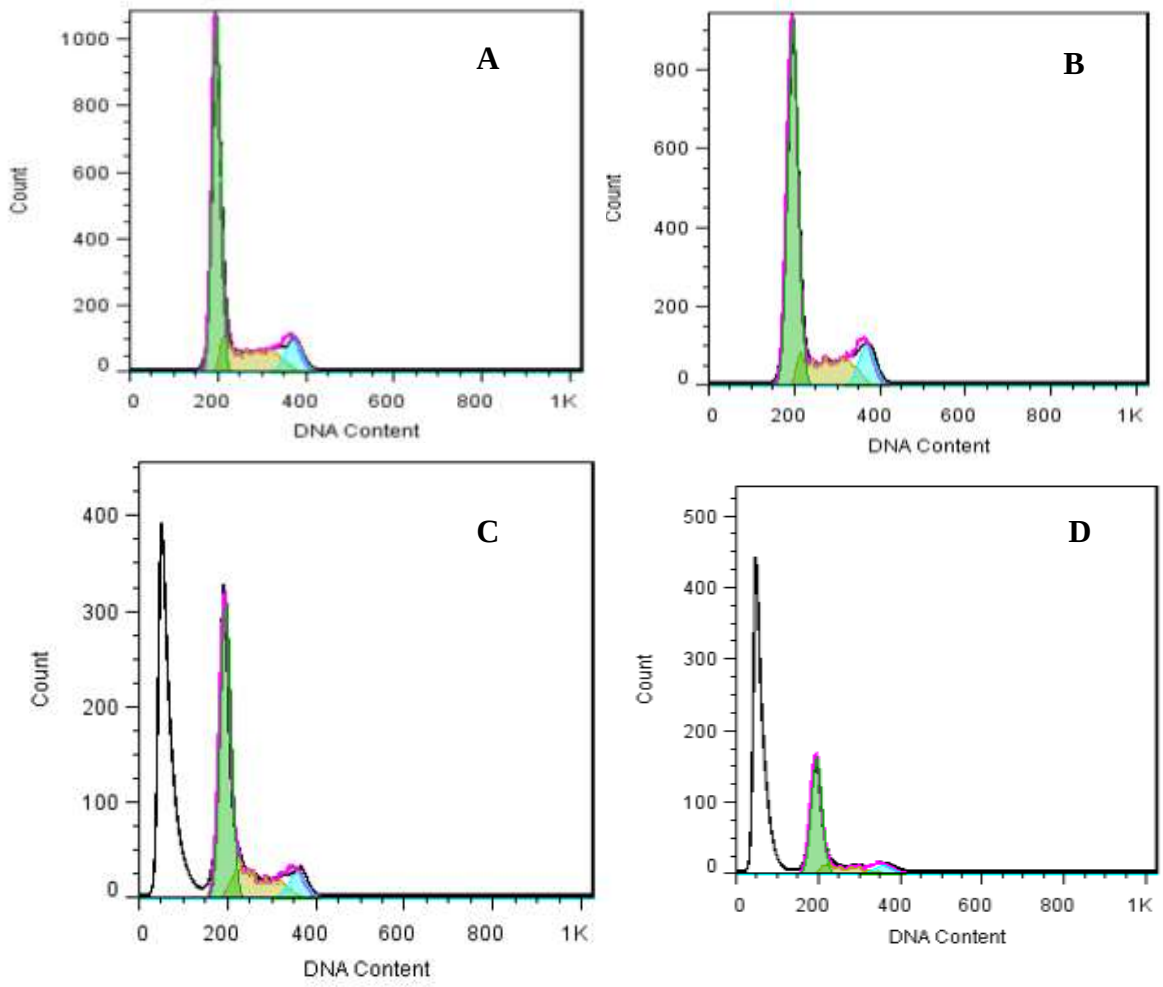


Fig. 2. Flow cytometric analysis of cells cycle distribution on MCF-7 and A549 cells: (A) treated with DMSO; (B) treated with sorafenib; (C) treated with **8l** against MCF-7; (D) treated with **8l** against A549

3. Conclusion

In conclusion, we have effectively synthesized two series of compounds bearing dihydrodipyrrolo[1,2-a:2',1'-c]pyrazines 8a-8s and their fused-pyridazine-dione analogues 10a-10q as promising cytotoxic agents. Their anti-proliferative activities against five human cancerous cell lines, including MCF7, HeLa, SW480, HepG2, and A549 were investigated, leading to identify compound 8l as potential candidate for further evaluations. This compound exhibited the IC_{50} values of 2.80±0.03 µM and 2.53±0.05 µM against MCF-7 and A549 cells, respectively. These figures were comparable to those of sorafenib, the reference drug in this study. Moreover, compound 8l induced apoptosis and arrested the cell cycle at the G0 phase, effectively stopping proliferation and triggering programmed cell death. Therefore, the present study revealed that these scaffolds have great potential for further structurally modifications to develop novel anti-cancer agents. Moreover, comprehensive studies on the mechanism of action of these compounds should be performed to further evaluate their anticancer efficacy.

4. Experimental

All chemicals were purchased from Merck (Germany) and were used without further purification. The reaction progress and the purity of

synthesized compounds were monitored by thin-layer chromatography (TLC) on silica gel 250-micron F254 plastic sheets; zones were detected visually under UV light (254 nm). Melting points were measured on an Electrothermal 9100 apparatus. 1H and 13C NMR spectra were measured (DMSO-d6 solution) with Bruker DRX-500 AVANCE (at 500.1 and 125.8 MHz) instruments. Chemical shifts were reported in parts per million (ppm), downfield from tetramethylsilane. Proton coupling patterns were described as singlet (s), doublet (d), triplet (t), and multiplet (m). Mass spectra were recorded on an Agilent Technologies (HP) 5973 mass spectrometer operating at an ionization potential of 70 eV.

4-1. General synthetic procedures

4-1-1. General synthetic procedure for the synthesis of 2-(1H-pyrrol-1-yl)ethan-1-amine 3

A solution containing 2-chloroethan-1-amine hydrochloride 2 (1 equiv.) and K2CO3 (1.5 equiv.) in DMF was heated at 80 °C for 30min. Then, pyrrole (1 equiv.) was added gradually, and resulting mixture was heated at the same temperature for further 12 hours. After completion of the reaction which was monitored by TLC, the mixture was cooled down to room temperature. Then, water was added to the mixture and extracted three times with EtOAc. The combined organic extracts were washed with brine, dried over Na2SO4 and then concentrated. The residue was purified by column chromatography to give the desired, pure 2-(1H-pyrrol-1-yl)ethan-1-amine 3.

4-1-2. General procedure for the synthesis of substituted phenylglyoxales (5a-s)

To the stirring solution containing selenium dioxide (1 equiv.) in the appropriate amount of dioxane and water at 50 °C, desired acetophenone 4 (1 equiv.) was added, and resulting blend was refluxed with continuous stirring for four hours. Afterwards, the hot solution was separated from the precipitated selenium, and the dioxane and water were removed through distillation using a short column. Therefore, arylglyoxal 5 remained and was used for further step without need to any purification process.

4-1-3. General procedure for the synthesis of dimethyl 1-substituted-5,6-dihydrodipyrrolo[1,2-a:2',1'-c]pyrazine-2,3-dicarboxylate (8a-s)

A mixture of N-ethyl amino pyrrole 3 (1 equiv.), arylglyoxal 5 (1 equiv.), and acetic acid (0.2 equiv.) in DCM was stirred at room temperature for one hour. After confirming completion of the reaction by TLC, a mixture of triphenylphosphine (0.5 equiv.) and dimethyl acetylene dicarboxylate (0.5 equiv.) in DCM were introduced into the mixture. Afterwards, the reaction mixture was stirred for further 10 min. The resulted mixture was then continued stirring at room temperature for an additional hour. Finally, the solvent was removed under reduced pressure, and crude product was purified using column chromatography to afford the pure, desired products 8a-8s in good yields.

Dimethyl-1-phenyl-5,6-dihydrodipyrrolo[1,2-a:2',1'-c]pyrazine-2,3-dicarboxylate (8a)

Green powder; yield: 70%; mp: 140-142 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ: 3.71 (s, 3H, CH_3), 3.84 (s, 3H, CH_3), 4.25 (t, 2H, *J*=5.8 Hz, CH_2), 4.79 (t, 2H, *J*=6.0 Hz, CH_2), 5.94 (dd, 1H, *J*=3.7, 1.4 Hz, H_b), 6.04 (dd, 1H, *J*=3.7, 2.7 Hz, H_a), 6.67 (dd, 1H, *J*=2.6, 1.4 Hz, H_c), 7.32-7.36 (m, 1H, H_{Ar}), 7.38 (t, 2H, *J*=7.9 Hz, H_{Ar}), 7.43 (d, 2H, *J*=7.9 Hz, H_{Ar}); ^{13}C NMR (125 MHz, $CDCl_3$): δ =43.4, 44.0, 51.9, 52.4, 107.2, 109.2, 117.8, 119.3, 120.8, 122.4, 125.1, 127.7, 127.9, 128.5, 130.0, 133.3, 161.1, 166.8; ESI-MS *m/z*: 350.1 $[M+H]^+$.

Dimethyl-1-(2-hydroxyphenyl)-5,6-dihydrodipyrrolo[1,2-a:2',1'-c]pyrazine-2,3-dicarboxylate) (8b)

Green powder; yield: 60% ; mp: 200-203 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ: 3.74 (s, 3H, CH_3), 3.85 (s, 3H, CH_3), 4.27 (q, 2H, *J*=5.2 Hz, CH_2), 4.65 (dt,

1H, *J*=13.2, 6.4 Hz, CH_2), 4.94 (dt, 1H, *J*=11.0, 5.0 Hz), 5.72 (dd, 1H, *J*=3.8, 1.6 Hz, H_b), 5.93 (s, 1H, OH), 6.04 (dd, 1H, *J*=4.0, 2.4 Hz, H_a), 6.65-6.68 (m, 1H, H_c), 6.95 (t, 1H, *J*=7.4 Hz, H_a), 7.06 (d, 1H, *J*=8.2 Hz, H_{Ar}), 7.21 (d, 1H, *J*=7.6 Hz, H_{Ar}), 7.31 (t, 1H, *J*=7.7 Hz, H_{Ar}); ^{13}C NMR (125 MHz, $CDCl_3$): δ= 43.5, 43.9, 52.0, 52.7, 107.7, 109.8, 113.1, 117.2, 118.7, 119.0, 120.1, 120.9, 121.1, 121.8, 129.1, 130.2, 132.2, 154.4, 161.0, 167.2; ESI-MS *m/z*: 366.12 $[M+H]^+$.

Dimethyl-1-(3-hydroxyphenyl)-5,6-dihydrodipyrrolo[1,2-a:2',1'-c]pyrazine-2,3-dicarboxylate (8c)

white powder; yield: 61%; mp: 138-140 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ: 3.72 (s, 3H, CH_3), 3.83 (s, 3H, CH_3), 4.21 (t, 2H, J=6.0 Hz, CH_2), 4.75 (t, 2H, J=5.9 Hz, CH_2), 5.61 (s, 1H, OH), 6.02 (d, 1H, *J*=3.9 Hz, H_b), 6.05 (s, 1H, H_a), 6.66 (s, 1H, H_c), 6.81 (d, 1H, *J*=7.8 Hz, H_{Ar}), 6.87 (s, 1H, H_{Ar}), 6.96 (d, 1H, *J*=7.3 Hz, H_{Ar}), 7.23 (t, 1H, *J*=7.7 Hz, H_{Ar}) ; ^{13}C NMR (125 MHz, $CDCl_3$): 43.3, 43.9, 51.9, 52.5, 107.4, 109.4, 114.8, 116.8, 117.7, 118.9, 120.9, 122.3, 125.0, 127.9, 129.7, 134.6, 155.9, 161.0, 167.1. ESI-MS *m/z*: 366.3 $[M+H]^+$.

Dimethyl-1-(o-tolyl)-5,6-dihydrodipyrrolo[1,2-a:2',1'-c]pyrazine-2,3-dicarboxylate (8d)

Green powder; yield: (70%); mp: 158-160 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ: 2.17 (s, 3H, CH_3), 3.65 (s, 3H, CH_3), 3.85 (s, 3H, CH_3), 4.25 (t, 2H, *J*=6.0 Hz, CH_2), 4.80-4.82 (m, 2H, CH_2), 5.49 (dd, 1H, *J*=3.8, 2.5 Hz, H_b), 6.01 (dd, 1H, *J*=3.7, 2.7 Hz, H_a), 6.65 (dd, 1H, *J*=2.6, 1.4 Hz, H_c), 7.19-7.23 (m, 2H, H_{Ar}), 7.27 (d, 2H, *J*=2.4 Hz, H-Ar); ^{13}C NMR (125 MHz, $CDCl_3$) δ: 20.1, 43.3, 44.0, 51.8, 52.1, 106.5, 109.5, 118.0, 118.6, 120.7, 122.6, 124.8, 125.8, 127.9, 128.1, 129.9, 130.9, 132.7, 138.1, 161.1, 166.3; ESI-MS *m/z*: 364.14 $[M+H]^+$.

Dimethyl-1-(m-tolyl)-5,6-dihydrodipyrrolo[1,2-a:2',1'-c]pyrazine-2,3-dicarboxylate (8e)

Cream powder; mp: 128-130 °C; yield (66%); ^{1}H NMR (500 MHz, $CDCl_3$) δ: 2.37 (s, 3H, CH_3), 3.72 (s, 3H, CH_3), 3.84 (s, 3H, CH_3), 4.25 (t, 2H, *J*=6.0 Hz, CH_2), 4.79 (t, 2H, *J*=5.9, Hz, CH_2), 5.97 (d, 1H, *J*=3.8 Hz, H_b), 6.06 (d, 1H, *J*=3.3 Hz, H_a), 7.15 (d, 1H, *J*=7.2 Hz, H_{Ar}), 7.22 (d, 1H, *J*=7.4 Hz, H_{Ar}), 7.27 (d, 2H, *J*=10.6 Hz, H_{Ar}); ^{13}C NMR (125 MHz, $CDCl_3$) δ: 21.5, 43.3, 44.0, 51.8, 52.3, 107.1, 109.2, 117.7, 119.4, 120.8, 122.5, 125.1, 127.0, 127.9, 128.3, 128.4, 130.6, 133.1, 137.9, 161.0, 166.8; ESI-MS *m/z*: 364.14 $[M+H]^+$.

12-(p-tolyl)-5,6,9,10-tetrahydropyrrolo[2'',1'':3',4']pyrazino[1',2':1,5]pyrrolo[2,3-d]pyridazine-8,11-dione (8f)

Green powder; mp: 164-166 °C; yield (68%); ^{1}H NMR (500 MHz, $CDCl_3$) δ: 2.39 (s, 3H, CH_3), 3.72 (s, 3H, CH_3), 3.83 (s, 3H, CH_3), 4.24 (t, 2H, *J*= 5.8 Hz, CH_2), 4.78 (d, 2H, *J*=5.8 Hz, CH_2), 5.98 (dd, 1H, *J*=3.6, 1.4 Hz, H_b), 6.02-6.09 (m, 1H, H_a), 6.66 (dd, 1H, *J*=2.8, 1.6 Hz, H_b), 7.19 (d, 2H, *J*=7.8 Hz, H_{Ar}), 7.32 (d, 2H, *J*=8 Hz, H_{Ar}); ^{13}C NMR (125 MHz, $CDCl_3$) δ: 21.4, 43.3, 43.9, 51.8, 52.3, 107.1, 109.1, 117.6, 119.3, 120.7, 122.5, 125.1, 127.9, 129.2, 129.8, 130.1, 137.3, 161.0, 166.9; ESI-MS *m/z*: 364.14 $[M+H]^+$.

Dimethyl-1-(3-methoxyphenyl)-5,6-dihydrodipyrrolo[1,2-a:2',1'-c]pyrazine-2,3-dicarboxylate (8g)

Green powder; mp: 138-140 °C; yield (73%); ^{1}H NMR (500 MHz, DMSO-d_6) δ: 3.64 (s, 3H, CH_3), 3.74 (s, 3H, CH_3), 3.77 (s, 3H, CH_3), 4.32 (t, 2H, *J*=5.6 Hz, CH_2), 4.66 (t, 2H, *J*=5.7 Hz, CH_2), 5.86 (dd,1H, *J*=3.7, 1.5 Hz, H_b), 5.98-6.01 (m, 1H, H_a), 6.88 (d,1H, *J*=2.2 Hz, H_c), 6.90-6.95 (m, 3H, H_{Ar}), 7.33 (t, 1H, *J*=7.9 Hz, H_{Ar}); ^{13}C NMR (125 MHz, DMSO-d_6): δ= 43.0, 43.2, 51.7, 52.0, 55.0, 106.1, 108.4, 113.0, 115.0, 117.4, 117.8, 121.2, 121.6, 121.7, 123.9, 126.9, 129.4, 134.3, 159.0, 160.0, 165.8; ESI-MS *m/z*: 380.14 $[M+H]^+$.

Dimethyl-1-(4-methoxyphenyl)-5,6-dihydrodipyrrolo[1,2-a:2',1'-c]pyrazine-2,3-dicarboxylate (8h)

Green powder; mp: 198-200 °C; yield (66%); ^{1}H NMR (500 MHz, $CDCl_3$) δ: 3.71 (s, 3H, CH_3), 3.84 (s, 3H, CH_3), 3.84 (s, 3H, CH_3), 4.24 (t, 2H, *J*=5.8 Hz, CH_2), 4.77 (t, 2H, *J*=5.8, CH_2), 5.96 (d, 2H, J=3.8 Hz, H_b), 6.03-6.06 (m, 1H, H_a), 6.66 (s, 1H, H_c), 6.92 (d, 2H, *J*=8.4 Hz, H_{Ar}), 7.35 (d, 2H, J=8.4 Hz, H_{Ar}); ^{13}C NMR (125 MHz, $CDCl_3$) δ: 43.3, 43.9, 51.8, 52.2, 55.3, 107.0, 109.2, 113.9, 117.6, 118.9, 120.7, 122.5, 125.1, 125.3, 128.0, 131.1, 159.1, 161.0, 166.9; ESI-MS *m/z*: 380.2$[M+H]^+$.

Dimethyl-1-(2-fluorophenyl)-5,6-dihydrodipyrrolo[1,2-a:2',1'-c]pyrazine-2,3-dicarboxylate (8i)

Green powder; mp: 138-140 °C; yield (62%); ^{1}H NMR (500 MHz, DMSO-d_6) δ: 3.61 (s, 3H, CH_3), 3.79 (s, 3H, CH_3), 4.32 (t, 2H, *J*=5.8 Hz, CH_2), 4.64 (t, 2H, *J*=5.8 Hz, CH_2), 5.60 (d, 1H, *J*=3.3 Hz, H_b), 5.96-6.03 (m, 1H, H_a), 6.92 (dd, 1H, *J*=2.6, 1.4 Hz, H_c) 7.27 (dt, 2H, *J*=14.9, 8.4 Hz, H_{Ar}), 7.34 (t, 1H, *J*=7.2 Hz, H_{Ar}), 7.45 (d, 1H, *J*=7.0 Hz, H_{Ar}); ^{13}C NMR (125 MHz, DMSO-d_6): δ= 43.1, 43.3, 51.8, 51.9, 105.5, 108.6, 110.9, 115.4, 115.6, 119.3, 120.6, 120.7, 121.0, 121.6, 122.9, 124.3, 127.4, 130.0, 130.1, 132.4, 158.9, 160.3, 160.8, 164.9; ESI-MS *m/z*: 368.12 $[M+H]^+$.

Dimethyl-1-(4-fluorophenyl)-5,6-dihydrodipyrrolo[1,2-a:2',1'-c]pyrazine-2,3-dicarboxylate (8j)

white powder; mp: 170-172 °C; yield (71%); ^{1}H NMR (500 MHz, $CDCl_3$) δ: 3.71 (s, 3H, CH_3), 3.84 (s, 3H, CH_3), 4.25 (t, 2H, *J*=5.9 Hz, CH_2), 4.78 (t, 2H, *J*=5.9 Hz, CH_2), 5.89 (dd, 1H, *J*=3.7, 0.7 Hz, H_b), 6.06 (dd, 1H, *J*=3.7, 2.6 Hz, H_a), 6.66-6.70 (m, 1H, H_c) 7.08 (t, 2H, *J*=8.8, H_{Ar}), 7.39 (dd, 2H, *J*=8.7, 5.5Hz, H_{Ar}); ^{13}C NMR (125 MHz, $CDCl_3$) δ: 43.4, 44.0, 51.9, 52.4, 107.0, 109.3, 115.4, 115.6, 118.0, 118.1, 120.9, 122.2, 125.0, 128.0, 129.2, 131.7, 131.8, 161.0, 161.5, 163.5, 166.6; ESI-MS *m/z*: 368.12 $[M+H]^+$.

Dimethyl-1-(3-chlorophenyl)-5,6-dihydrodipyrrolo[1,2-a:2',1'-c]pyrazine-2,3-dicarboxylate (8k)

Light green powder; mp: 140-143 °C; yield (79%); ^{1}H NMR (500 MHz, $CDCl_3$) δ: 3.73 (s, 3H, CH_3), 3.85 (s, 3H, CH_3), 4.26 (t, 2H, *J*=5.9 Hz, CH_2), 4.78 (t, 2H, *J*=5.9 Hz, CH_2), 5.95 (dd, 1H, *J*=3.8, 1.4 Hz, H_b), 6.07 (t, 1H, *J*=3.2 Hz, H_a), 6.67-6.71 (1H, m, H_c), 7.30-7.34 (m, 3H, H_{Ar}), 7.44 (s, 1H, H_{Ar}); ^{13}C NMR (125 MHz, $CDCl_3$) δ: 43.4, 44.0, 52.0, 52.4, 107.2, 109.4, 117.7, 118.2, 121.1, 122.0, 124.8, 127.9, 127.9, 128.3, 129.7, 130.0, 134.2, 135.2, 161.0 166.4; ESI-MS *m/z*: 384.09 $[M+H]^+$.

Dimethyl-1-(4-chlorophenyl)-5,6-dihydrodipyrrolo[1,2-a:2',1'-c]pyrazine-2,3-dicarboxylate (8l)

Green powder; mp: 195-198 °C; yield (62%); ^{1}H NMR (500 MHz, $CDCl_3$) δ: 3.72 (s, 3H, CH_3), 3.84 (s, 3H, CH_3), 4.25 (t, 2H, *J*=5.9 Hz, CH_2), 4.77 (t, 2H, *J*=5.9 Hz, CH_2), 5.94 (dd, 1H, *J*=3.8, 1.4 Hz, H_b), 6.06 (dd, 1H, *J*=3.7, 2.6 Hz, H_a), 6.68 (dd, 1H, *J*=2.5, 1.4 Hz, H_c), 7.33-7.39 (4H, m, H_{Ar}); ^{13}C NMR (125 MHz, $CDCl_3$) δ : 43.4, 43.9, 51.9, 52.4, 107.1, 109.3, 117.8, 118.1, 121.0, 122.1, 124.8, 127.9, 128.7, 131.4, 131.8, 135.6, 160.9, 166.5. ESI-MS *m/z*: 386.08 $[M+H]^+$.

Dimethyl-1-(3-bromophenyl)-5,6-dihydrodipyrrolo[1,2-a:2',1'-c]pyrazine-2,3-dicarboxylate (8m)

Green powder; mp: 145-147 °C; yield (73%); ^{1}H NMR (500 MHz, DMSO-d_6) δ: 3.65 (s, 3H, CH_3), 3.78 (s, 3H, CH_3), 4.32 (t, 2H, *J*=5.9 Hz, CH_2), 4.64 (t, 2H, *J*=5.9 Hz, CH_2), 5.77 (dd, 1H, *J*=3.7, 1.5 Hz, H_b), 6.01 (dd, 1H, *J*=3.8, 2.6 Hz, H_a), 6.90 (dd, 1H, *J*=2.6, 1.4 Hz, H_c), 7.34 (d, 1H, *J*=7.7 Hz, H_{Ar}), 7.39 (t, 1H, J=7.8, H_{Ar}), 7.50 (s, 1H, H_{Ar}), 7.57 (d, 1H,

J=7.9 Hz, H_{Ar}),:^{13}C NMR (125 MHz, DMSO-d_6) δ: 43.0, 43.3, 51.8, 52.0, 105.8, 108.5, 115.9, 118.5, 120.9, 121.4, 121.8, 123.3, 127.0, 128.6, 130.3, 130.6, 132.0, 135.4, 160.0, 165.5; ESI-MS *m/z*: 428.04 $[M+H]^+$.

Dimethyl-1-(4-bromophenyl)-5,6-dihydrodipyrrolo[1,2-a:2',1'-c]pyrazine-2,3-dicarboxylate (8n)

Green powder; mp:210-214 °C; yield (60%); ^{1}H NMR (500 MHz, $CDCl_3$) δ: 3.72 (s, 3H, CH_3), 3.84 (s, 3H, CH_3), 4.25 (t, 2H, *J*=5.9 Hz, CH_2), 4.78 (t, 2H, *J*=5.9 Hz, CH_2), 5.94 (dd, 1H, *J*=3.7, 1.3 Hz, H_b), 6.07 (dd, 1H, *J*=3.5, 2.9 Hz, H_a), 6.68 (dd, 1H, *J*=2.6, 1.5 Hz, H_c), 7.31 (d, 2H, *J*=8.5 Hz, H_{Ar}), 7.51 (d, 2H, *J*=8.5 Hz, H_{Ar}); ^{13}C NMR (125 MHz, DMSO-d_6) δ: 43.4, 44.0, 52.0, 52.4, 107.2, 109.4, 117.9, 118.2, 121.0, 121.9, 122.1, 124.8, 127.9, 131.7, 132.3, 161.0, 166.6.

Dimethyl-1-(3-nitrophenyl)-5,6-dihydrodipyrrolo[1,2-a:2',1'-c]pyrazine-2,3-dicarboxylate (8o)

Green powder; mp:180-182 °C; yield (73%); ^{1}H NMR (500 MHz, $CDCl_3$) δ: 3.74 (s, 3H, CH_3), 3.86 (s, 3H, CH_3), 4.28 (t, 2H, *J*=5.0 Hz, CH_2), 4.79 (t, 2H, *J*=5.0 Hz, CH_2), 5.86 (dd, 1H, *J*=3.7, 1.3 Hz, H_b), 6.06 (dd, 1H, *J*=3.5, 2.9 Hz, H_a), 6.71 (t, 1H, *J*=5.0 Hz, H_c), 7.57 (t, 1H, *J*=10.0 Hz, H_{Ar}), 7.79 (d, 1H, *J*=10.0 Hz, H_{Ar}), 8.20 (d, 1H, *J*=5.0 Hz, H_{Ar}), 8.33 (s, 1H, H_{Ar}); ^{13}C NMR (125 MHz, $CDCl_3$) δ: 43.5, 44.0, 52.1, 52.4, 107.0, 109.5, 116.5, 118.9, 121.4, 121.6, 122.6, 124.4, 125.1, 128.0, 129.4, 135.3, 136.4, 148.4, 160.9, 166.1. ESI-MS *m/z*: 396.1 $[M+H]^+$.

Dimethyl-1-(4-nitrophenyl)-5,6-dihydrodipyrrolo[1,2-a:2',1'-c]pyrazine-2,3-dicarboxylate (8p)

Dark orang powder; mp: 220-223 °C; yield (68%); ^{1}H NMR (500 MHz, DMSO-d_6) δ: 3.73 (s, 3H, CH_3), 3.86 (s, 3H, CH_3), 4.29 (t, 2H, *J*=5.9 Hz, CH_2), 4.79 (t, 2H, *J*=5.9 Hz, CH_2), 5.94 (dd, 1H, *J*=3.8, 1.4 Hz, H_b), 6.08 (dd, 1H, *J*=3.8, 2.6 Hz, H_a), 6.69-6.74 (m, 1H, Hc),7.63 (d, 2H, *J*=8.7 Hz, H_{Ar}), 8.25 (d, 2H, *J*=8.6 Hz, H_{Ar}); ^{13}C NMR(125 MHz, DMSO-d_6) δ: 43.3, 43.9, 51.8, 52.3, 55.3, 107.0, 109.2, 113.9, 117.6, 118.9, 120.7, 122.5, 125.1, 125.3, 128.0, 131.1, 159.1, 161.0, 166.8; ESI-MS *m/z*: 395.11 $[M+H]^+$.

Dimethyl-1-(4-hydroxy-3-methoxyphenyl)-5,6-dihydrodipyrrolo[1,2-a:2',1'-c]pyrazine-2,3-dicarboxylate (8q)

Light green powder; mp: 215-217 °C; yield (80%); ^{1}H NMR (500 MHz, $CDCl_3$) δ: 3.73 (s, 3H, CH_3), 3.83 (s, 3H, CH_3), 3.84 (s, 3H, CH_3), 4.24 (t, 2H, *J*=5.8 Hz, CH_2), 4.78 (t, 2H, *J*=5.8 Hz, CH_2), 5.73 (s, 1H, OH), 6.02-6.04 (m, 1H, H_b), 6.06 (dd, 1H, *J*=3.8, 2.3 Hz, H_a), 6.67 (dd, 1H, *J*=2.7, 1.4 Hz, H_c), 6.93 (s, 2H, H_{Ar}), 6.96 (s, 2H, H_{Ar}); ^{13}C NMR (125 MHz, $CDCl_3$) δ: 43.3, 44.0, 51.8, 52.4, 56.0, 107.2, 109.2, 112.7, 114.5, 117.5, 119.1, 120.8, 122.4, 125.2, 128.0, 145.3, 146.4, 107.0, 167.0; ESI-MS *m/z*: 396.2 $[M+H]^+$.

Dimethyl-1-(2,4-dichlorophenyl)-5,6-dihydrodipyrrolo[1,2-a:2',1'-c]pyrazine-2,3-dicarboxylate (8r)

Dark green powder; mp: 161-163 °C; yield (72%); ^{1}H NMR (500 MHz, $CDCl_3$) δ: 3.69 (s, 3H, CH_3), 3.86 (s, 3H, CH_3), 4.22-4.31 (m, 2H, CH_2), 4.77 (t, 2H, *J*=5.9 Hz), 5.64 (dd, 1H, *J*=3.4, 1.8 Hz, H_b), 6.07 (dd, 1H, *J*=3.3 Hz, H_a), 6.66-6.74 (m, 1H, H_c), 7.25-7.35 (m, 2H, H_{Ar}), 7.51 (d, 1H, *J*= 1.8 Hz, H_{Ar}); ^{13}C NMR (125 MHz, $CDCl_3$) δ: 43.3, 43.8, 51.9, 52.1, 106.8, 109.5, 115.1, 119.2, 120.9, 121.9, 123.7, 127.1, 128.0, 129.5, 131.2, 133.4, 134.5, 135.9, 161.1, 165.4; ESI-MS *m/z*: 418.05 $[M+H]^+$.

Dimethyl-1-(3,4,5-trimethoxyphenyl)-5,6-dihydrodipyrrolo[1,2-a:2',1'-c]pyrazine-2,3-dicarboxylate (8s)

Green powder; mp: 164-167 °C; yield (60%); 3.75 (s, 3H, CH_3), 3.80 (s, 6H, $2CH_3$), 3.83 (s, 3H, CH_3), 3.89 (s, 3H, CH_3), 4.25 (t, 2H, *J*=5.8 Hz, CH_2), 4.78

(t, 2H, *J*=5.9 Hz, CH_2), 6.07 (dd, 1H, *J*=3.6, 2.4 Hz, H_b), 6.13 (dd, 1H, *J*=3.8, 1.4 Hz, H_a), 6.65-6.70 (3H, m, H_c, 2 H_{Ar}); ^{13}C NMR (125 MHz, $CDCl_3$) δ: 43.3, 43.9, 51.8, 52.4, 56.2, 61.0, 107.0, 107.4, 109.2, 117.6, 118.9, 120.9, 122.2, 125.1, 127.8, 128.5, 137.5, 152.1, 160.8, 167.0; ESI-MS *m/z*: 440.16 $[M+H]^+$.

4-1-4. General procedure for the synthesis of 12-substituted-5,6,9,10 tetrahydropyrrolo [2'',1'':3',4']pyrazino[1',2':1,5]pyrrolo[2,3-d]pyridazine-8,11-dione (10a-10q)

Phthalate **8a-8s** (1 equiv.) was dissolved in ethanol, followed by the addition of hydrazine hydrate **9** (1 equiv.). The resulting mixture was refluxed for 10 hours. After cooling to room temperature and removing the solvents under reduced pressure, the crude product was triturated in water, filtered, and dried under vacuum to obtain cyclic hydrazide **10a-q** with great to excellent yields.

11-hydroxy-12-phenyl-5,6-dihydropyrrolo[2'',1'':3',4']pyrazino[1',2':1,5] pyrrolo[2,3-d]pyridazin-8(9H)-one (10a)

White powder; mp: 350-353 °C; yield (75%);^{1}H NMR (500 MHz, DMSO-d_6): 4.34 (t, 2H, *J*=5.5 Hz, CH_2), 4.89 (t, 2H, *J*=5.7 Hz, CH_2), 5.77-5.82 (m, 1H, H_b), 5.97 (t, 1H, *J*=3.2 Hz, H_a), 6.39 (d, 1H, *J*=2.2 Hz, H_c) 7.31-7.36 (m, 1H, H_{Ar}), 7.39 (t, 2H, *J*=7.1 Hz, H_{Ar}), 7.42 (d, 2H, *J*=6.8 Hz, H_{Ar}); ^{13}C NMR (125 MHz, DMSO-d_6) δ : 42.6, 43.3, 106.3, 108.3, 113.3, 117.1, 121.6, 121.7, 124.8, 126.9, 127.5, 128.4, 130.9, 133.5, 152.6, 153.5; ESI-MS *m/z*: 318.11 $[M+H]^+$.

11-hydroxy-12-(3-hydroxyphenyl)-5,6-dihydropyrrolo[2'',1'':3',4']pyrazino [1',2':1,5]pyrrolo[2,3-d]pyridazin-8(9H)-one (10b)

White powder; mp: 341-343 °C; yield (72%); ^{1}H NMR (500 MHz, DMSO-d_6) δ: 4.34 (t, 2H, *J*=5.9 Hz, CH_2), 4.86 (t, 2H, *J*=5.8 Hz, CH_2), 5.84 (d, 1H, *J*=3.7 Hz, H_b), 5.99 (t, 1H, *J*=3.2 Hz, H_a), 6.74 (d, 1H, *J*=8 Hz, H_c), 6.81 (d, 2H, *J*=7.9 Hz, H_{Ar}), 6.93 (s, 1H, H_{Ar}), 7.17 (t, 1H, 7.5 Hz, H_{Ar}); ^{13}C NMR (125 MHz, DMSO-d_6) δ: 42.6, 43.3, 106.6, 108.3, 113.4, 113.9, 116.5, 117.0, 117.9, 121.6, 124.6, 128.4, 128.7, 131.4, 134.7, 156.6.

11-hydroxy-12-(o-tolyl)-5,6-dihydropyrrolo[2'',1'':3',4']pyrazino[1',2':1,5] pyrrolo[2,3-d]pyridazin-8(9H)-one (10c)

Yellow powder; mp: 331-333 °C; yield (77%); ^{1}H NMR (500 MHz, DMSO-d_6) δ: 2.06 (s,3H, CH_3), 4.34 (t, 2H, *J*=5.9 Hz, CH_2), 4.82-4.96 (m, 2H, CH_2), 5.38 (d, 1H, *J*=3.7 Hz, H_b), 5.94 (t, 1H, *J*=3.1 Hz, H_a), 6.91 (s, 1H, H_c), 7.20 (m, 2H, H_{Ar}), 7.29 (m, 2H, H_{Ar}); ^{13}C NMR (125 MHz, DMSO-d_6) δ: 19.9, 42.6, 43.3, 106.0, 108.6, 117.9, 121.6, 121.8, 124.6, 125.3, 127.4, 128.6, 129.5, 130.5, 131.0, 133.6, 134.2, 136.2, 137.5, 155.4; ESI-MS *m/z*: 332.13 $[M+H]^+$.

11-hydroxy-12-(m-tolyl)-5,6-dihydropyrrolo[2'',1'':3',4']pyrazino[1',2':1,5] pyrrolo[2,3-d]pyridazin-8(9H)-one (10d)

Cream powder; mp: 331-333 °C; yield (71%); ^{1}H NMR (500 MHz, DMSO-d_6) δ: 2.37 (s, 3H, CH_3), 4.34 (t, 2H, *J*=5.8 Hz, CH_2), 4.88 (t, 2H, *J*=5.8 Hz, CH_2), 5.78 (d,1H, *J*=3.8 Hz, H_b), 5.97 (t, 1H, *J*=3.2 Hz, H_a), 6.89-6.99 (m, 1H, H_c), 7.15 (d, 1H, *J*= 7.3 Hz, H_{Ar}) 7.20 (d, 1H, *J*= 7.4 Hz, H_{Ar}), 7.23 (s, 1H, H_{Ar}), 7.28 (t, 1H, *J*=7.5 Hz, H_{Ar}); ^{13}C NMR (125 MHz, DMSO-d_6) δ: 21.0, 42.6, 43.3, 106.3, 108.3, 113.4, 117.2, 121.7, 124.7, 127.5, 127.6, 128.4, 128.9, 131.5, 133.4, 136.4, 152.7, 153.6; ESI-MS *m/z*: 332.13 $[M+H]^+$.

11-hydroxy-12-(p-tolyl)-5,6-dihydropyrrolo[2'',1'':3',4']pyrazino[1',2':1,5] pyrrolo[2,3-d]pyridazin-8(9H)-one (10e)

White powder; mp: 340-343 °C; yield (71%); ^{1}H NMR (500 MHz, DMSO-d_6) δ: 2.37 (s, 3H, CH_3), 2.17 (t, 2H, *J*=5.7 Hz, CH_2), 4.86 (t, 2H, *J*=5.5 Hz, CH_2), 5.81 (dd, 1H, *J*= 3.8, 1.4 Hz, H_b), 5.98 (t, 1H, *J*= 3.2 Hz, H_a), 6.93 (t, 1H, *J*=2.0 Hz, H_c), 7.20 (d, 2H, *J*=7.8 Hz, H_{Ar}), 7.29 (d, 2H, *J*=7.9 Hz, H_{Ar}); ^{13}C

NMR (125 MHz, DMSO-d_6) δ: 20.9, 42.7, 43.3, 106.5, 108.4, 116.0, 116.9, 121.5, 121.8, 124.2, 128.2, 128.8, 130.2, 130.7, 136.1, 151.8, 153.2; ESI-MS *m/z*: 332.13 $[M+H]^+$.

11-hydroxy-12-(3-methoxyphenyl)-5,6-dihydropyrrolo[2'',1'':3',4']pyrazino [1',2':1,5]pyrrolo[2,3-d]pyridazin-8(9H)-one (10f)

Yellow powder; mp: 380-383 °C; yield (75%); ^{1}H NMR (500 MHz, DMSO-d_6) δ: 3.75 (s, 3H, OCH_3), 4.34 (t, 2H, *J*=5.8 Hz, CH_2), 4.87 (t, 2H, *J*=4.9 Hz, CH_2), 5.85 (d, 1H, *J*=5.85 Hz, H_b), 5.99 (m, 1H, H_a), 6.90-6.95 (m, 2H, H_c, H_{Ar}), 6.95-7.02 (m, 2H, H_{Ar}), 7.30 (t, 1H, *J*=7.8 Hz, H_{Ar}); ^{13}C NMR (125 MHz, DMSO-d_6) δ: 42.7, 43.3, 55.0, 106.6, 108.4, 112.5, 113.1, 116.6, 117.2, 121.5, 121.8, 123.2, 124.6, 128.6, 134.8, 152.5, 153.4, 158.6, 168.7; ESI-MS *m/z*: 348.12 $[M+H]^+$.

11-hydroxy-12-(4-methoxyphenyl)-5,6-dihydropyrrolo[2'',1'':3',4']pyrazino [1',2':1,5]pyrrolo[2,3-d]pyridazin-8(9H)-one (10g)

White powder; mp: 337-338 °C; yield (73%); ^{1}H NMR (500 MHz, DMSO-d_6) δ: 3.81 (s, 3H, OCH_3), 4.34 (t, 2H, *J*= 4.9 Hz, CH_2), 4.87 (t, 2H, *J*=5.4 Hz, CH_2), 5.83 (dd, 1H, *J*=3.7, 1.6 Hz, H_b), 5.99 (t, 1H, *J*= 3.1 Hz, H_a), 6.90-6.93 (m, 1H, H_c), 6.96 (d, 2H, *J*=8.0 Hz, H_{Ar}), 7.33 (d, 2H, *J*=8.4 Hz, H_{Ar}); ^{13}C NMR (125 MHz, DMSO-d_6) δ: 42.6, 43.3, 55.0, 106.4, 108.4, 113.0, 113.1, 117.1, 121.6, 121.7, 124.5, 125.4, 128.6, 131.9, 152.6, 153.2, 158.3; ESI-MS *m/z*: 348.12 $[M+H]^+$.

11-hydroxy-12-(4-fluorophenyl)-5,6-dihydropyrrolo[2'',1'':3',4']pyrazino [1',2':1,5]pyrrolo[2,3-d]pyridazin-8(9H)-one (10h)

White powder; m.p.: 340-342 °C; yield 0.241g (72%); ^{1}H NMR (500 MHz, DMSO-d_6) δ: 4.35 (t, 2H, *J*=5.7 Hz, CH_2), 4.87 (t, 2H, *J*=5.9 Hz, CH_2), 5.80 (d, 1H, *J*=3.7 Hz, H_b), 6.00 (t, 1H, *J*=3.1 Hz, H_a), 6.94 (d, 1H, *J*=2.5 Hz, H_c), 7.22 (t, 2H, *J*=10.0 Hz, H_{Ar}), 7.45(dd, 2H, *J*=10.0, 5.0 Hz, H_{Ar}); ^{13}C NMR (125 MHz, DMSO-d_6) δ: 42.7, 43.3, 106.4, 108.4, 112.0, 114.5 (d, *J*=21.2), 114.6, 117.1, 121.3, 121.9, 124.5, 125.7, 129.6, 132.8 (d, *J*=7.5 Hz), 152.4, 155.5, 162.4, 164.4; ESI-MS *m/z*: 336.2 $[M+H]^+$.

11-hydroxy-12-(3-chlorophenyl)-5,6-dihydropyrrolo[2'',1'':3',4']pyrazino [1',2':1,5]pyrrolo[2,3-d]pyridazin-8(9H)-one (10i)

White powder; mp: 368-370 °C; yield (78%); ^{1}H NMR (500 MHz, DMSO-d_6) δ: 4.34 (t, 2H, *J*=5.0 Hz, CH_2), 4.89 (t, 2H, *J*=5.1 Hz, CH_2), 5.81-5.84 (m, 1H, H_b), 6.01 (d, 1H, *J*=3.1 Hz, H_a), 6.94 (d, 1H, *J*=2.6 Hz, H_c), 7.38-7.44 (m, 3H, H_{Ar}), 7.47 (s, 1H, H_{Ar}); ^{13}C NMR (125 MHz, DMSO-d_6) δ: 42.6, 43.3, 106.2, 108.4, 111.8, 117.2, 121.3, 121.9, 125.2, 126.7, 128.2, 129.3, 129.6, 130.6, 132.1, 135.8, 148.3, 152.7; ESI-MS *m/z*: 352.07 $[M+H]^+$.

11-hydroxy-12-(4-chlorophenyl)-5,6-dihydropyrrolo[2'',1'':3',4']pyrazino [1',2':1,5]pyrrolo[2,3-d]pyridazin-8(9H)-one (10j)

White powder; mp: 335-337 °C; yield (70%); ^{1}H NMR (500 MHz, DMSO-d_6) δ: 4.36 (t, 2H, *J*=5.0 Hz, CH_2), 4.86 (t, 2H, *J*=5.0 Hz, CH_2), 5.74 (s, 1H), 5.81-5.83 (m, 1H, H_b), 6.01 (1H, t, *J*=5.0 Hz, H_a), 6.95-6.96 (1H, m, H_c), 7.41-7.45 (4H, m, H_{Ar}); ^{13}C NMR (125 MHz, $CDCl_3$) δ: 42.7, 43.3, 106.6, 108.5, 116.0, 117.0, 121.2, 122.1, 124.5, 125.4, 127.7, 131.8, 132.2, 132.7, 152.7, 154.9; ESI-MS *m/z*: 352.07 $[M+H]^+$.

11-hydroxy-12-(3-bromophenyl)-5,6-dihydropyrrolo[2'',1'':3',4']pyrazino [1',2':1,5]pyrrolo[2,3-d]pyridazin-8(9H)-one (10k)

White powder; mp: 368-370 °C; yield (80%); ^{1}H NMR (500 MHz, DMSO-d_6) δ: 4.36 (d, 2H, *J*=10.0 Hz, CH_2), 4.88 (t, 2H, *J*=10.0 Hz, CH_2), 5.82 (dd, 1H, *J*=5.0, 1.5 Hz, H_b), 6.02 (t, 1H, J= 5.0 Hz, H_a), 6.97 (s, 1H, H_c), 7.35-7.38 (m,1H, H_{Ar}), 7.46 (d, 1H, J=5.0 Hz, H_{Ar}), 7.55 (d, 1H, *J*=10.0 Hz, H_{Ar}), 7.60 (s, 1H, H_{Ar}); ^{13}C NMR (125 MHz, DMSO-d_6) δ: 42.7, 43.3, 106.3, 108.5, 111.4, 114.4, 120.7, 121.2,

122.1, 122.8, 128.7, 129.7 (2), 130.0, 133.4, 135.9, 152.4, 153.2; ESI-MS (*m/z*): 396.02 $[M+H]^+$.

11-hydroxy-12-(4-bromophenyl)-5,6-dihydropyrrolo[2'',1'':3',4']pyrazino [1',2':1,5]pyrrolo[2,3-d]pyridazin-8(9H)-one (10l)

White powder; mp: 330-332 °C; yield (72%); ^{1}H NMR (500 MHz, DMSO-d_6) δ: 4.35 (dd, 2H, *J*=5.0 Hz, CH_2), 4.85 (t, 2H, *J*=5.0 Hz, CH_2), 5.81-5.84 (m, 1H, H_b), 6.01 (1H, t, *J*= 5.0 Hz, H_a), 6.95 (s, 1H, H_c) 7.38 (d, 2H, *J*=10.0 Hz, H_{Ar}), 7.58 (d, 2H, *J*=5.0 Hz, H_{Ar}); ^{13}C NMR (125 MHz, DMSO-d_6) δ: 42.8, 43.3, 106.7, 108.6, 111.5, 116.9, 120.4, 121.1, 122.2, 124.4, 129.0, 130.6, 132.6, 133.1, 148.6, 152.2. ESI-MS (*m/z*): 396.1 $[M+H]^+$.

11-hydroxy-12-(3-nitrophenyl)-5,6-dihydropyrrolo[2'',1'':3',4']pyrazino [1',2':1,5]pyrrolo[2,3-d]pyridazin-8(9H)-one (10m)

Yellow powder; mp: 330-332 °C; yield (76%); ^{1}H NMR (500 MHz, DMSO-d_6) δ: 4.38 (t, 2H, *J*=5.0 Hz, CH_2), 4.90 (t, 2H, *J*=5.0 Hz, CH_2), 5.88 (d, 1H, *J*=5.0 Hz, H_b), 6.01-6.02 (m, 1H, H_a), 6.99 (s, 1H, H_c), 7.69-7.72 (m, 1H, H_{Ar}), 7.95 (d, 1H, *J*=10.0 Hz, H_{Ar}), 8.22 (d, 1H, *J*=5.0 Hz, H_{Ar}), 8.29 (s, 1H, H_{Ar}); ^{13}C NMR (125 MHz, DMSO-d_6) δ: 42.7, 43.3, 106.3, 108.6, 117.0, 119.3, 120.9, 121.8, 122.2, 124.9, 125.4, 125.9, 129.1, 135.1, 137.7, 47.2, 151.3, 153.5; ESI-MS (*m/z*): 363.1 $[M+H]^+$.

11-hydroxy-12-(4-nitrophenyl)-5,6-dihydropyrrolo[2'',1'':3',4']pyrazino [1',2':1,5]pyrrolo[2,3-d]pyridazin-8(9H)-one (10n)

Yellow powder; mp: 330-332 °C; yield (70%); ^{1}H NMR (500 MHz, DMSO-d_6) δ: 4.38 (t, 2H, *J*=5.0 Hz, CH_2), 4.89 (t, 2H, *J*=7.5 Hz, CH_2), 5.92-5.93 (m, 1H, H_b), 6.02-6.03 (m, 1H, H_a), 7.00 (s, 1H, H_c), 7.75 (2H, d, J=10.0 Hz, H_{Ar}), 8.26 (d, 2H, *J*=5.0 Hz, H_{Ar}); ^{13}C NMR (125 MHz, DMSO-d_6) δ: 42.8, 43.2, 106.8, 108.6, 111.0, 117.0, 120.8, 122.4, 122.7, 125.1, 128.7, 131.4, 132.0, 132.2, 132.3, 133.1, 140.9, 146.2, 152.1, 153.3; ESI-MS (*m/z*): 363.2 $[M+H]^+$.

11-hydroxy-12-(4-hydroxy-3-methoxyphenyl)-5,6-dihydropyrrolo[2'',1'':3',4'] pyrazino[1',2':1,5]pyrrolo[2,3-d]pyridazin-8(9H)-one (10o)

Yellow powder; mp: 288-290 °C; yield (82%); ^{1}H NMR (500 MHz, DMSO- d_6) δ: 3.71 (s, 3H, OCH_3), 4.34 (t, 2H, *J*=5.0 Hz, CH_2), 4.86 (s, 2H, CH_2), 5.90-5.91 (m, 1H, H_b), 6.00 (m, 1H, H_a), 6.78-6.81 (m, 2H, $H_{Ar,}$ H_c), 6.94 (d, 2H, *J*=5.0 Hz, H_{Ar}); ^{13}C NMR (125 MHz, DMSO-d_6) δ: 43.3, 44.0, 51.8, 52.4, 56.0, 107.2, 109.2, 112.7, 114.5, 117.5, 119.1, 120.8, 122.4, 123.0, 125.0, 125.2, 128.0, 145.3, 146.4, 161.0, 167.0; ESI-MS (*m/z*): 364.12 $[M+H]^+$.

11-hydroxy-12-(2,4-dichlorophenyl)-5,6-dihydropyrrolo[2'',1'':3',4'] pyrazino[1',2':1,5]pyrrolo[2,3-d]pyridazin-8(9H)-one (10p)

White powder; mp: 360-362 °C; yield (70%); ^{1}H NMR (500 MHz, DMSO-d_6) δ: 4.34 (t, 2H, *J*=5.0 Hz, CH_2), 4.89 (t, 2H, *J*=5.0 Hz, CH_2), 5.82-5.83 (1H, m, H_b), 6.01-6.02 (m 1H, H_a), 6.94 (d, 1H, *J*=5.0 Hz, H_c), 7.40-7.42 (2H, m, H_{Ar}), 7.47 (s, 1H, H_{Ar}); ^{13}C NMR (125 MHz, DMSO-d_6) δ: 42.6, 43.3, 106.4, 108.8, 121.0, 122.1, 127.0, 128.6, 128.7, 128.8, 131.4, 131.5, 132.0, 133.0, 133.1, 134.1, 135.4, 156.8; ESI-MS (*m/z*): 386.1 $[M+H]^+$.

11-hydroxy-12-(3,4,5-trimethoxyphenyl)-5,6-dihydropyrrolo[2'',1'':3',4'] pyrazino[1',2':1,5]pyrrolo[2,3-d]pyridazin-8(9H)-one (10q)

White powder; mp: 328-333 °C; yield (70%); ^{1}H NMR (500 MHz, DMSO-d_6) δ: 3.73 (s, 9H, $3CH_3$), 4.36 (t, 2H, *J*=5.0 Hz, CH_2), 4.88 (t, 2H, *J*=5.0 Hz, CH_2), 5.97-5.98 (m, 1H, H_b), 6.03-6.04 (1H, m, H_a), 6.74 (s, 2H, H_c, H_{Ar}), 6.95 (s, 1H, H_{Ar}); ^{13}C NMR (125 MHz, $CDCl_3$) δ: 42.7, 43.3, 55.9, 60.1, 106.7, 108.4, 113.2, 117.0, 121.5, 121.8, 124.4, 128.6, 136.6, 152.1.

4-2. MTT assay

Cytotoxicity of the compounds was measured by detecting the ability of cells in transforming MTT to a purple formazan dye. Five different cell lines including MCF7, HeLa, SW480, HepG2, and A549, provided from the National Cell Bank of Iran (Pastor Institute, Tehran, Iran). The required materials and reagents were purchased from Sigma. After the cell cultur in RPMI-1640 medium and DMEM containing 10% FBS (Gibco, Milano, Italy), the suspension of HEPG-2 (7000) and other cells (5000) were poured into 96 well plate and incubated in a humidified incubator containing 5% CO2 at 37 °C for 24 hours. Subsequently, a solution of the compounds in DMSO were added to these plates and incubated for 48 hours. After incubation, the solution of 5% 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was added to all the wells and incubation was continued for another 4 hours. The record of color intensity of the formazan solution was at 570 nm by Bio-Rad microplate reader (Model 680) which reflects the cell growth condition [37].

4-3. Apoptosis inducing Analysis

For determination of apoptosis induction, the best concentration (IC_{50}) of the most active compounds were selected and evaluated on MCF-7 and A549 cell lines. After incubation of the in a 6-well plate at 37 ° C for 24 hours, they treated with selected compounds at their IC_{50} concentration for 48 h. Then, cells were trypsinized, rinsed with Phosphate buffered saline (PBS) and then centrifuged at 1200 rpm for 3 minutes. After this, the binding buffer (500 µL) was added to the resulting cells, followed by addition and mixing with Annexin V-APC and PI (5 µL). After this, the samples were incubated in dark for 10 to 15 minutes at room temperature, and then the cellular analysis was measured by flow cytometer (FACS Calibur Bectone-Dickinson).

4-4. Cell-Cycle Analysis

The selected compounds were examined on MCF-7 and A549 cells at IC_{50} concentration for 48 h. After trypsinization and rinsing with PBS, the cells were centrifuged at 1200 rpm for 5 minutes and then incubated with PBS and fixed in ice-cooled 70% ethanol. After washing with PBS, the cells were resuspended in RNase A (0.1 mg/ml) and incubated for 5h. Then, staining with PI (50 mg/ml) and incubation for 15 minutes were performed. By using Novocyte flow cytometer (ACEA Biosciences) the analysis was done and the calculation of cell cycle distributions was done by NovoExpress 1.1.0 software.

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