Design, Synthesis, and Pharmacological Assay of Novel Compounds Based on Pyridazine Moiety as Potential Antitumor Agents

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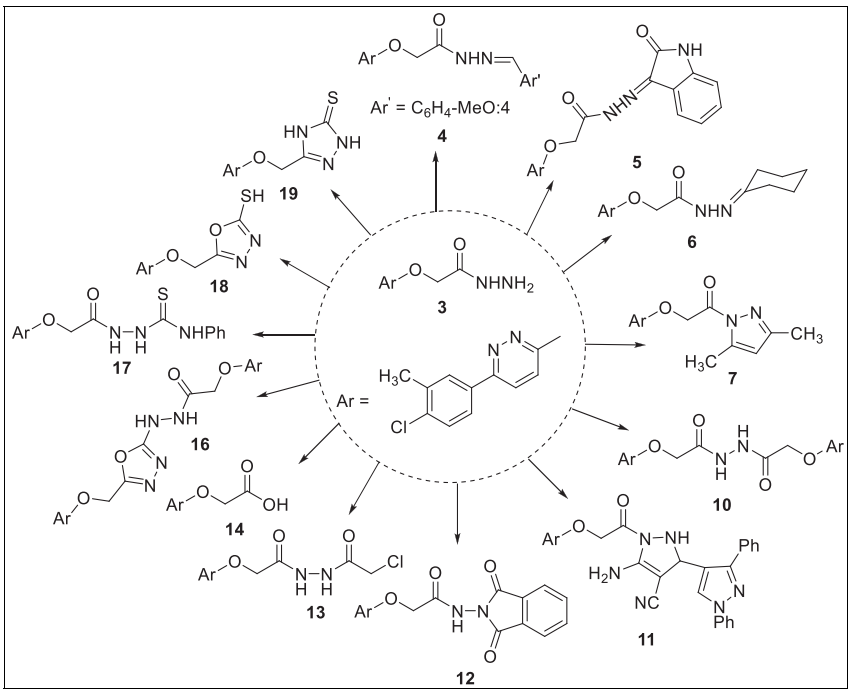


In an endeavor to develop antitumor agents, we made a credible survey regarding synthesis, structure, and pharmacological assay of novel pyridazine derivatives, so that 2-((6-(4-chloro-3-methylphenyl)pyridazin-3-yl)oxy)acetohydrazide **3** was utilized as scaffold to build novel compounds **4**–**19** by reaction with various electrophilic reagents, followed by determination and explanation atropisomerism phenomena and tauomerism ratio such as keto-enol and lactam–lactim tautomers for some synthesized compounds. *In vitro*, these compounds were screened for antitumor efficacy versus two cell lines, namely, hepatocellular carcinoma and mammary gland breast cancer, by using MTT assay. Among the examined compounds, compound **16** was exhibited promising potent activity (IC_{50} = 8.67 ± 0.7 μM) versus HepG2 cell line. Meanwhile, compounds **3** and **16** were manifested the very highest efficacy (IC_{50} = 5.68 ± 0.6 and 9.41 ± 0.9 μM) versus MCF-7 cell line.

INTRODUCTION

Over the last decades, pyridazine core [1] is considered as crucial structure in medicinal chemistry as pharmaceuticals [2,3], agrochemistry [4–6] as herbicides [7], material science [6,8,9], and supermolecular chemistry [10], where it has a vast variety of biological activities, for example, antidepressant, antifungal, antibacterial [11,12], antituberculosis [13], antiviral, anticancer [14], antihypertensive [15], anti-inflammatory [16], cardiovascular disorders [17], vasorelaxant activities [18,19], SGLT2 inhibitors [20], and anti-HRV [21] (Fig. 1).

Indeed, during our literature survey, it is clearly noticeable that, pyridazine nucleus constructed by variant methods [18,20,22–25], one of them involving reaction of β-aroyl acrylic acid and/or β-aroyl propionic acid with hydrazine monohydrate, which has been previously prepared in our lab [26,27].

As a part of our studies on incorporating of acetohydrazide to pyridazine moiety to exploit the nucleophlicity of the nitrogen atom of hydrazide group to attack on various electrophilic centers to build novel compounds in order to assay their particular biological potentials.

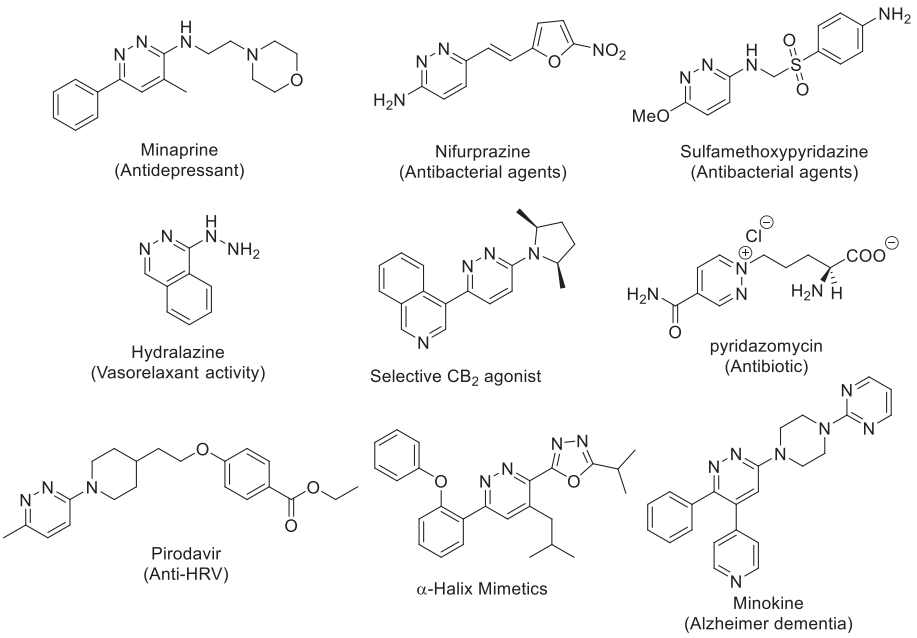


Figure 1. Pharmaceuticals containing pyridazine nucleus.

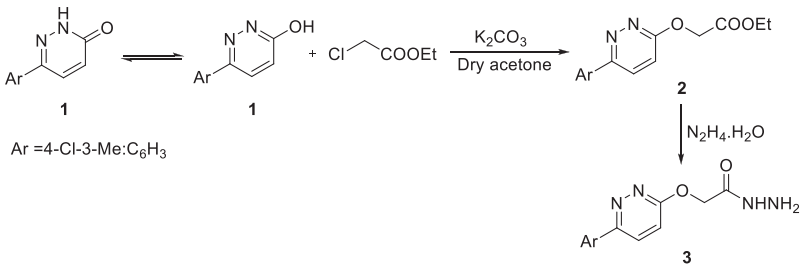
RESULTS AND DISCUSSION

As a continuation of our efforts to study the reactivity of acetohydrazide which connecting with pyridazine ring as a scaffold to develop new pharmacologically active compounds. In this article, the targeted pyridazine synthesized according to the common manners as exemplified in Scheme 1. Thus, 2-((6-(4-chloro-3-methylphenyl)pyridazin-3-yl)oxy) acetohydrazide **3** [28] was formed from the reaction of 6-(4-chloro-3-methylphenyl)pyridazin-3(2*H*)-one **1** [26] with ethyl chloroacetate in dry acetone as a solvent in the presence of anhydrous K_2CO_3 to afford **2**, followed by hydrazinolysis.

At the outset, some obvious trends will be observed in this article to exploit the acetohydrazide **3** as a precursor to construct novel compounds based on pyridazine moiety. Therefore, condensation of **3** with *p*-anisaldehyde in dioxane afforded the Schiff base derivative **4** (Scheme 2).

The predictable structure of compound **4** was explicated by ^{1}H NMR spectrum that emerged only one singlet peak (exchangeable with D_2O) at δ = 11.59 ppm compatible with one NH proton, two doublet peaks at δ = 8.17 and 8.12 ppm compatible with H_b, a singlet peak at δ = 7.98 ppm compatible with methine proton H_e, a doublet peak at δ = 7.90 ppm compatible with $H_{c'}$, two doublet peaks at δ = 7.75 and 7.72 ppm compatible with H_c, a doublet peak at δ = 7.65 ppm compatible with $2H_g$, two doublet peaks at δ = 7.53 and 7.48 ppm compatible with H_d, a doublet peak at δ = 7.12 ppm compatible with H_a, a doublet peak at δ = 7.00 ppm compatible with $2H_f$, a singlet peak at δ = 5.28 ppm compatible with CH_2, a singlet peak at δ = 3.80 ppm compatible with OCH_3, and two singlet peaks at δ = 2.39 and 2.37 ppm compatible with CH_3. The appearance of extra signals which indicated the existence of **4** in two conformations **4A** and **4B** (as atropisomers), because of hindered rotation of the

Scheme 1. The strategy for synthesis of compound **3**.



Scheme 2. Synthesis of Schiff base derivatives **4**–**6**.

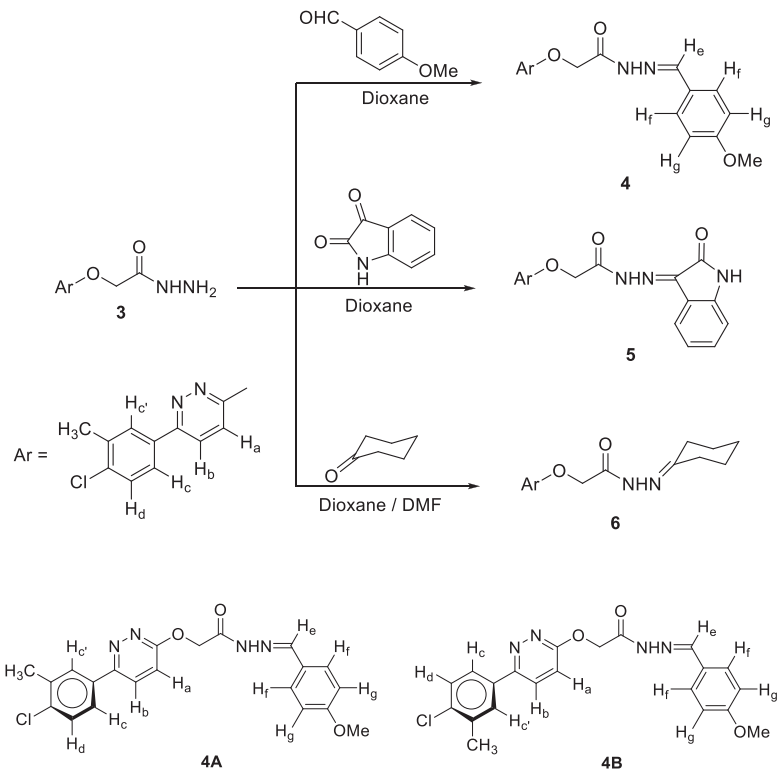


Figure 2. The atropisomerism phenomena for compound **7**.

aryl ring about the pyridazine moiety through pivot bond as designed in the succeeding text in Figure 2.

Similarly, condensation of **3** with isatin is easily to afford 2-((6-(4-chloro-3-methylphenyl)pyridazin-3-yl)oxy)-*N*′-(2-oxoindolin-3-ylidene) acetohydrazide **5** (Scheme 2).

Manifestly, the condensed structure of **5** was ascertained from the IR spectrum that obtained two absorption bands at 1727 and 1696 cm^{-1} compatible with two carbonyl groups. As a clue for the proposed structure of **5**, ^1H NMR spectrum of compound **5** emerged the existence of two singlet peaks at 12.68 and 11.26 ppm corresponding to two NH protons beside the appearance of extra four aromatic protons in aromatic region corresponding to indolinone moiety. Lactam–lactim tautomerism in ratio 72:28, the higher ratio of the lactam tautomer compared with the lactim tautomer is due to their relative thermodynamic stabilities in DMSO, whereas lactam form stabilized by the formation of intramolecular H-bonding chelated in six-membered ring (Fig. 3).

In the context, refluxing of compound **3** with cyclohexanone in a mixture of dioxane and dimethylformamide (DMF) awarded the condensed product **6** as the sole product in moderate yield (Scheme 2). At analyzing ^1H NMR of Schiff base **6** in $CHCl_3$ as a solvent, it manifested a singlet peak at δ = 9.08 ppm compatible with deshielded one NH proton and the peak of NH_2 protons was absent. Additionally, ^1H NMR displayed the presence of two multiplet peaks at δ 2.36–2.24 and 1.71–1.62 ppm corresponding to five CH_2 protons of cyclohexylidene ring.

Heterocyclization of compound **3** with acetyl acetone awarded the pyrazole derivative **7** [28] (Scheme 3). In particular, ^1H NMR spectrum of **7** exhibited a singlet peak at δ = 6.45 ppm compatible with olefinic proton of

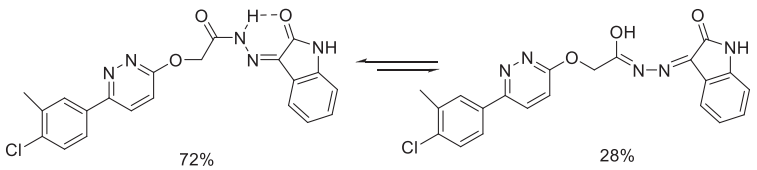
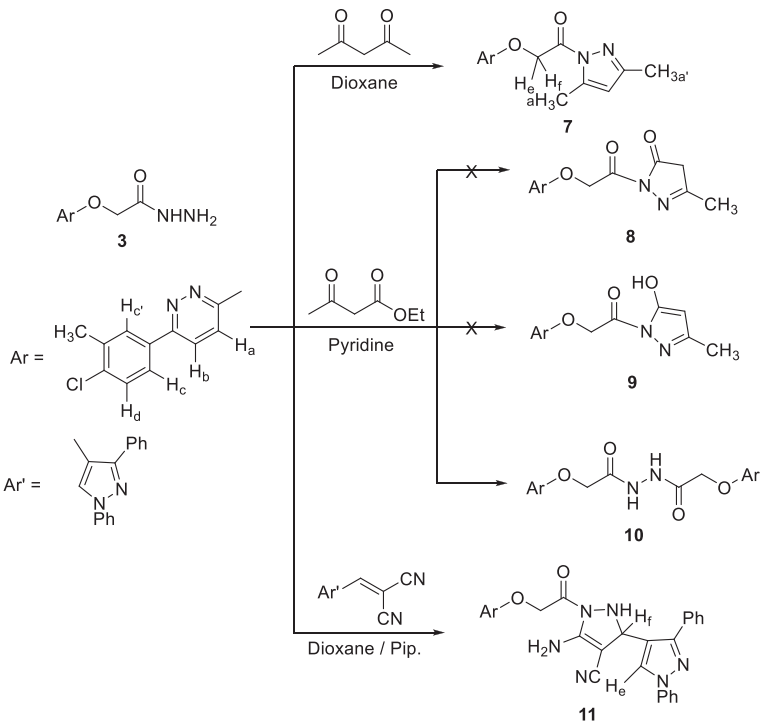


Figure 3. The lactam–lactim tautomers of compound **5**.

Scheme 3. Synthetic pathway to compounds **7**–**11**.



pyrazole ring beside two singlet peaks at 2.01 and 1.74 ppm compatible with CH_{3a} and $CH_{3a'}$ protons of pyrazole ring, respectively.

Indeed, reaction of hydrazide **3** with β-ketoester such as ethyl acetoacetate in dry pyridine awarded 2-((6-(4-chloro-3-methylphenyl) pyridazin-3-yl)oxy)-*N*′-(2-((6-(4-chloro-3-methylphenyl)pyridazin-3-yl)oxy)acetyl) acetohydrazide **10** instead of **8** and **9** [29] (Scheme 3). As evidence for the unexpected structure **10**, ^{1}H NMR spectrum (in DMSO) canceled the conception of formation of compounds **8** and/or **9** due to the lack of the singlet signals of CH_2 protons and olefinic proton of 5-pyrazolinone and pyrazole rings, respectively. While ^{1}H NMR spectrum emerged broad singlet peak at δ = 9.68 ppm (exchangeable with D_2O) compatible with two NH protons, five peaks at δ = 8.11–7.05 ppm compatible with 10 aromatic protons, and a singlet peak at δ = 2.36 ppm compatible with two CH_3 protons. Furthermore, the compound **10** existed as lactam–lactim tautomers in the ratio 13:87 (Fig. 4), due to the appearance of broad singlet peak at δ = 10.35 (exchangeable with D_2O) corresponding to two OH protons for lactam tautomer and two singlet peaks at δ = 4.90 ppm (for lactam tautomer) and δ = 4.84 ppm (for lactim tautomer) corresponding to four OCH_2 protons. The tremendous ratio of the lactim tautomer is due to its higher stability because of its more conjugated pattern as shown in Figure 4.

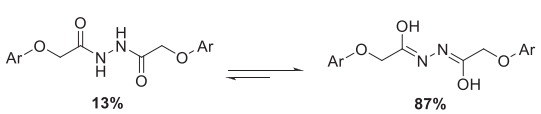


Figure 4. The lactam–lactim tautomers of compound **10**.

We envisioned that the structure of compound **10** was constructed through the succeeding mechanistic scenario (Scheme 4).

The structure of compound **10** and its mechanism were also elucidated laboratory by detection of 5-methyl-2,4-dihydro-3*H*-pyrazol-3-one as the side product in the resulting mixture by thin-layer chromatography (TLC) after separation of the unexpected product **10**.

Refluxing a mixture of carbohydrazide **3** with 2-((1,3-diphenyl-1*H*-pyrazol-4-yl)methylene)malononitrile in dioxane including small amount of piperidine as a base catalyzed afforded pyrazole derivative **11** (Scheme 3). The foreseeable structure of **11** was unequivocally ascertained from spectroscopic and elemental analyses. In DMSO solution, the ^{1}H NMR spectrum of **11** displayed a singlet peak at δ = 5.02 ppm compatible with H_f proton, beside two signals at 8.66, 3.44 ppm compatible with NH and NH_2 protons (both exchangeable with D_2O), respectively. Moreover, the ^{1}H NMR of **11** emerged that its comprising of a diasteromeric mixture of keto-enol tautomers in the ratio of 74:26 (Fig. 5). The intramolecular chelation of H-bonding in six-membered ring explained

Scheme 4. A plausible mechanistic pathway to compound **10**.

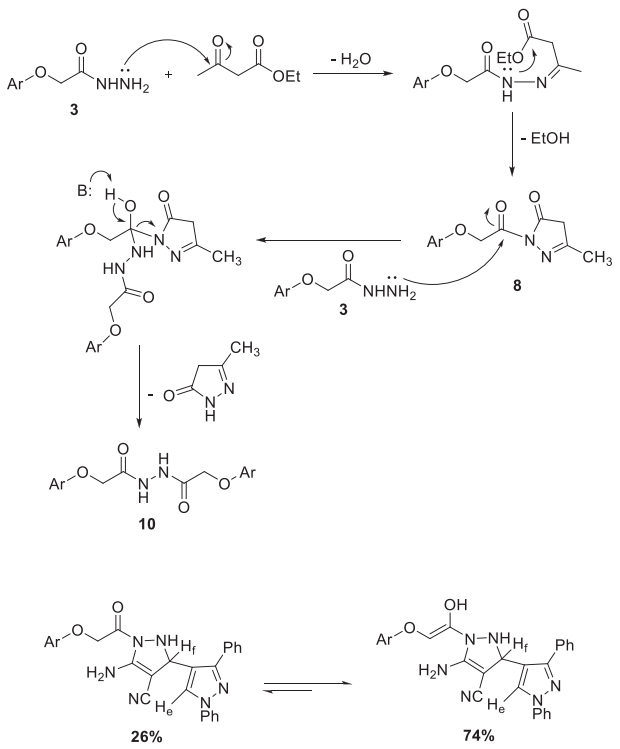


Figure 5. The keto-enol tautomers of compound **11**.

the higher proportion of enol tautomer of compound **11** as shown in the succeeding text in Figure 6, as it manifested a broad and a singlet peak at δ = 11.61 and 5.23 ppm compatible with OH and olefinic protons, respectively.

The phthalimido derivative **12** was commenced by treatment of **3** with phthalic anhydride in boiling n-butanol as a solvent (Scheme 5). The structure of compound **12** was elaborated by their spectroscopic data and elemental analysis. Obviously, the IR spectrum of **12** displayed $\upsilon_{(C=O)}$ at 1790, 1736, 1708, and 1656 cm^{-1}. Moreover, ^1H NMR spectrum obtained at δ = 11.10 ppm a singlet peak compatible with one NH proton beside that the peak of NH_2 protons was lacked.

Stirring of carbohydrazide **3** with chloroacetyl chloride in DMF at ambient temperature to afford smoothly 2-chloro-*N*′-(2-((6-(4-chloro-3-methylphenyl)pyridazin-3-yl) oxy)acetyl)acetohydrazide **13** (Scheme 5). After profound investigation of ^1H NMR spectrum of **13** which showed

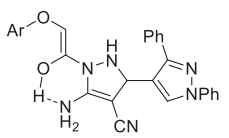


Figure 6. Intramolecular chelation in compound **11** (enol tautomer).

up a singlet peak at δ = 4.12 ppm characteristic to CH_2 protons, that coming from incorporation of chloroacetyl group *via* tetrahedral mechanism.

Refluxing of acetohydrazide derivative **3** in formic acid for 12 h afforded the hydrolyzed acetic acid derivative **14** (Scheme 5). Firstly, compound **14** was elucidated by IR spectrum, while it remarked a band at 1710 cm^{-1} compatible with C═O of acid. Secondly, ^1H NMR spectrum exhibited the existence of peak at 13.13 ppm (exchangeable with D_2O) corresponding to OH proton of acid.

Incredibly, when **3** was subjected to reflux with triethyl orthoformate, furnished the 1,3,4-oxadiazole derivative **16** (Scheme 5). The undesired structure of **16** was conspicuously ascertained by elemental analysis and different spectroscopic techniques. ^1H NMR spectrum of **16** showed at δ = 10.03 and 8.38 ppm two peaks exchangeable with D_2O compatible with two NH protons, at δ = 8.10–7.05 ppm a multiplet peak compatible with 10 aromatic protons, at δ = 5.09–4.78 ppm a multiplet peak compatible with two CH_2 protons, and at δ = 2.37 and 2.27 ppm two singlet peaks compatible with two CH_3 protons.

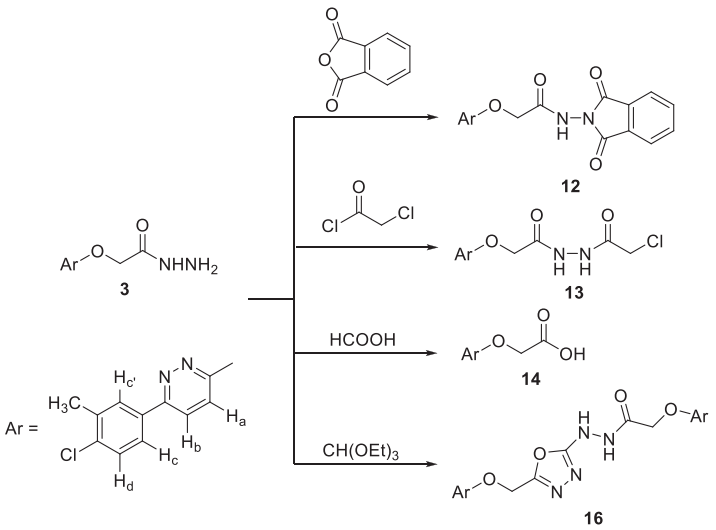
Mechanistically, the formation of **16** initiated from attacking of NH_2 of hydrazide that prompted as a nucleophile on the electrophilic carbon center of triethyl orthoformate to form ethyl formohydrazonate derivative **15** as an intermediate, followed by attacking of another molecule of carbohydrazide **3** and then finished by oxidation after intramolecular 1,5-*endo*-trig cyclization as shown in Scheme 6.

Refluxing a mixture of compound **3** with phenyl isothiocyanate in DMF as a solvent furnished 2-(2-((6-(4-chloro-3-methylphenyl)pyridazin-3-yl)oxy)acetyl)-*N*-phenylhydrazine-1-carbothioamide **17** (Scheme 7). ^1H NMR spectrum of the carbothioamide derivative **17** was clearly exhibited the presence of three peaks at δ 10.60, 10.36, and 9.65 ppm corresponding to three deshielded NH protons.

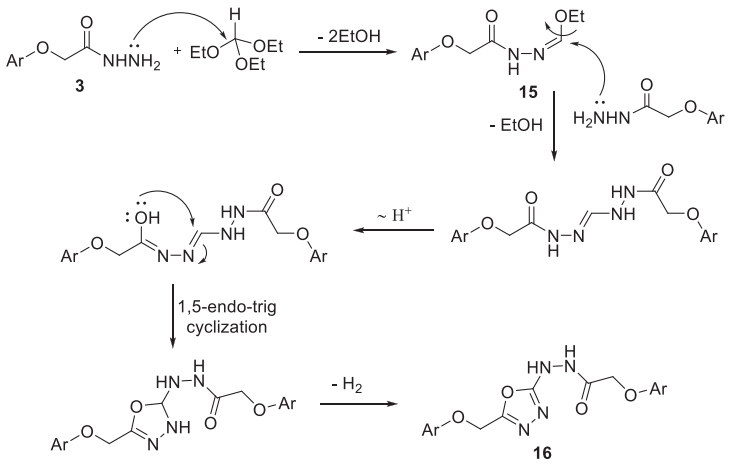
Curing a mixture of compound **3** with carbon disulfide in dioxane as a solvent under alkaline condition (aq. KOH) to reflux for 24 h afforded a mixture from 1,3,4-oxadiazole-2-thiol derivative **18** and the hydrolyzed product **14** (Scheme 7). This mixture was separated by treatment with Na_2CO_3 solution followed by filtration, and the residue was considerable as 1,3,4-oxadiazole-2(3*H*)-thione derivative **18**, while the mother liquor was acidified to get the hydrolyzed product **14.**

While 2,4-dihydro-3*H*-1,2,4-triazole-3-thione derivative **19** was emanated from refluxing a mixture of compound **3** with ammonium thiocyanate and concentrated HCl in aqueous solution for 30 min (Scheme 7), the spectral and elemental analyses for the compounds **18** and **19** are in keeping with their chemical structures.

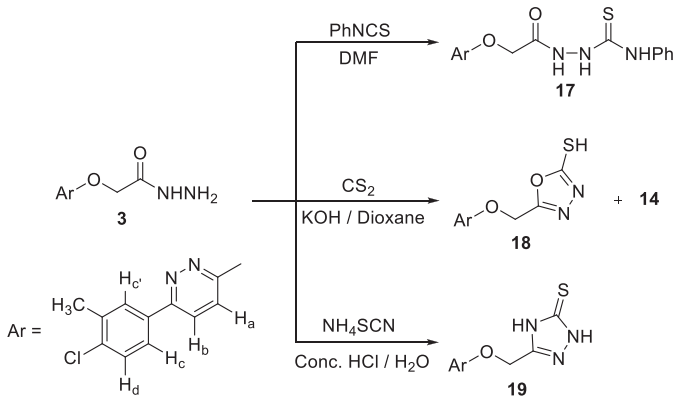
Scheme 5. Synthetic pathway to compounds **12**–**16**.



Scheme 6. A plausible mechanistic pathway to compound **16**.



Scheme 7. Synthetic pathway to compounds **17**–**19**.



PHARMACOLOGY

***In vitro* antitumor evaluation**. Evaluation of the antitumor activity *in vitro* for the synthesized compounds was employed by utilizing MTT assay [30,31] versus hepatocellular carcinoma (HepG2) and mammary gland breast cancer (MCF-7) as two human tumor cell lines. Doxorubicin was chosen as the reference anticancer drug. The inhibitory activities IC_{50} (μM) of compounds **3**–**19** are expressed in Table 1 and Figure 7.

As inspection of the data in Table 1 and Figure 7, which revealed that compound **16** has the best potent efficacy (IC_{50} < 10 μM) versus HepG2 cell line. Meanwhile, compounds **3** and **16** were manifested promising potent activities (IC_{50} < 10 μM) versus MCF-7 cell line in comparison with doxorubicin. For HepG2 cell line, the strong cytotoxic efficacy was remarked by compounds **3** and **6** that showed the percentage viability of IC_{50} at 13.91 ± 1.1 and 19.80 ± 1.6 μM, respectively. Meanwhile, compounds **7**, **11**, **13**, **17**, **18**, and **19** revealed moderate activity, whereas the rest of compounds exhibited weak activities. Meanwhile, the efficacy versus MCF-7 cell line emerged that compounds **6** and **7** have the strong percentage viability IC_{50} at 11.50 ± 1.0 and 18.34 ± 1.4 μM, respectively. Meanwhile, compounds **11**, **13**, **18**, and **19** revealed moderate activities, and the other screened compounds emerged weak activities.

Structure–activity relationships. Regardless of incorporation of 1,3,4-oxadiazole moiety beside carbohydrazide group to pyridazine ring as in compound **16** improved the antitumor efficacy toward two cell lines, namely, HepG2 and MCF-7. Unfortunately, the incorporation of other groups or heterocyclic moieties to pyridazine ring decreased the antitumor efficacy versus both the HepG2 and MCF-7 cells.

In an effort to manifest the structure–activity relationships' information, the parent carbohydrazide **3** showed more potency than other synthesized compounds except compound **16** against HepG2, which indicated that NH_2 group of carbohydrazide **3** increased the hydrophobicity character.

On the basis of the structural resemblance between the pyrazole, 1,3,4-oxadiazole, and 1,2,4-triazole rings in compounds **7**, **18**, and **19**, respectively, as depicted in Figure 8, its analogues have been evaluated *in vitro* for antitumor efficacy. The structure–activity relationships within these series are approximate.

Table 1

Antitumor activity of some new compounds versus HepG2 and MCF-7cells.

Compounds	*In vitro* cytotoxicity IC_{50} (μM)[a]	
	HepG2	MCF-7
DOX	4.50 ± 0.3	4.17 ± 0.2
3	13.91 ± 1.1	5.68 ± 0.6
4	55.19 ± 3.2	60.32 ± 3.7
5	72.96 ± 3.9	52.44 ± 3.4
6	19.80 ± 1.6	11.50 ± 1.0
7	25.73 ± 1.9	18.34 ± 1.4
10	69.12 ± 3.5	73.80 ± 4.2
11	43.84 ± 2.8	38.67 ± 2.5
12	91.65 ± 4.8	84.26 ± 4.7
13	49.38 ± 3.0	41.54 ± 2.8
14	62.72 ± 3.5	67.48 ± 3.9
16	8.67 ± 0.7	9.41 ± 0.9
17	83.45 ± 4.3	75.03 ± 4.5
18	28.65 ± 2.1	31.39 ± 2.3
19	36.07 ± 2.4	22.95 ± 1.7

DOX, doxorubicin.
[a]IC_{50} (μM): 1–10 (very strong); 11–20 (strong); 21–50 (moderate); 51–100 (weak); and above 100 (non-cytotoxic).

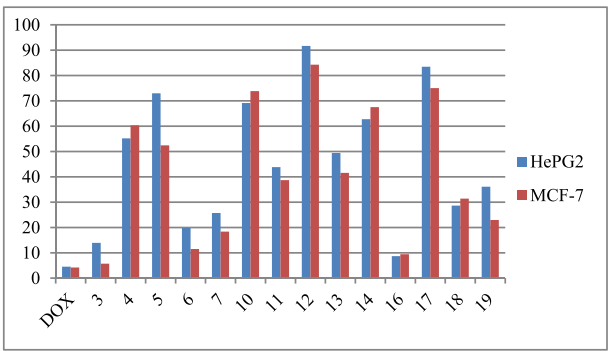


Figure 7. Antitumor activity of some new compounds versus HepG2 and MCF-7 cells. [Color figure can be viewed at wileyonlinelibrary.com]

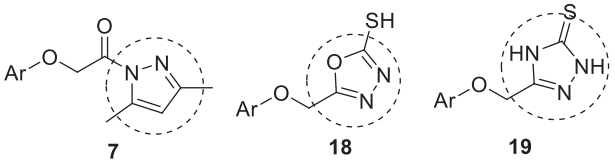


Figure 8. Bioisosteric relationship between five-membered rings heterocyclic.

CONCLUSION

In summary, in order to develop efficacious antitumor agents, we have designed, synthesized, and characterized novel structures based on pyridazine moiety, as the reaction of 2-((6-(4-chloro-3-methylphenyl)pyridazin-3-yl) oxy)acetohydrazide **3** with various electrophilic reagents such as aldehyde, ketones, β-diketone, β-ketoester, arylidene malononitrile, phthalic anhydride, chloroacetyl chloride, formic acid, triethyl orthoformate, phenyl isothiocyanate, carbon disulfide, and ammonium thiocyanate, followed by determination and explanation

atropisomerism phenomena and tauomerism ratio such as keto-enol and lactam–lactim tautomers for some synthesized compounds. Evaluation of their antitumor activities *in vitro* versus HepG2 and MCF-7 cell lines by MTT assay. Compound **16** was exhibited promising potent efficacy versus HepG2 cell line. Meanwhile, compounds **3** and **16** were manifested the very highest efficacy versus MCF-7 cell line.

EXPERIMENTAL

All melting points without correction were measured on Griffin and Georgy melting point apparatus. The IR spectra were obtained on the Nicolet iS10 FT-IR spectrometer using KBr Wafer technique. ^{1}H NMR spectra were made on a Varian Gemini 300 MHz utilizing tetramethylsilane as the reference and chemical shifts are explained in δ-scale. EI-MS were performed on a Schimadzu-GC–MS operating at 70 eV. Elemental analyses were proceeded by Perkin-Elmer 2400 CHN elemental analyzer. Biological activity was implemented at the drugs department, Faculty of pharmacy, Mansoura University, Egypt. The homogeneity and purity of the synthesized compounds was controlled by TLC (using TLC aluminum sheets silica gel F_{254} [Merck]).

2-((6-(4-Chloro-3-methylphenyl)pyridazin-3-yl)oxy) acetohydrazide 3 [28]. A mixture of ethyl 2-((6-(4-chloro-3-methylphenyl)pyridazin-3-yl)oxy)acetate **2** (1 g, 3.2 mmol) and hydrazine hydrate (0.16 mL) in ethanol (10 mL) was heated under reflux for 12 h. After cooling the reaction mixture, the separated solid was boiled with petroleum ether (bp 80–100°C) filtered off, dried fully, and recrystallized from ethanol/dioxane to give **3** as white crystals; mp: 220–222°C (Lit. mp: 210–212°C) [28], yield: 50%. IR (KBr, υ/cm^{-1}): 3318, 3252, 3120 (NH, NH_2), 2996, 2923 (CH_3, CH_2), 1669 (C═O), 1653 (C═N). ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm): 9.31 (s, 1H, NH, exchangeable with D_2O), 8.08–8.05 (d, 1H, Ar—H, H_b, J = 9.9 Hz), 7.87–7.86 (d, 1H, Ar—H, $H_{c'}$, J_m = 1.8 Hz), 7.74–7.71 (d, d, 1H, Ar—H, H_c, J_o = 8.4 Hz, J_m = 2.1 Hz), 7.51–7.48 (d, 1H, Ar—H, H_d, J = 8.4 Hz), 7.06–7.02 (d, 1H, Ar—H, H_a, J = 9.9 Hz), 4.72 (s, 2H, OCH_2), 4.27 (s, 2H, NH_2, exchangeable with D_2O), 2.39 (s, 3H, CH_3). MS *m/z* (%): 292 (M^+; 14.39), 261 (100), 233 (43.29), 220 (4.78), 206 (16.99), 183 (8.53), 162 (18.08), 142 (8.41) 127 (8.87), 101 (6.88), 52 (68.58). *Anal*. Calcd for $C_{13}H_{13}N_4O_2Cl$ (292.07): C, 53.34; H, 4.48; N, 19.14; Cl, 12.11. Found: C, 53.26; H, 4.54; N, 19.19; Cl, 12.02.

2-((6-(4-Chloro-3-methylphenyl)pyridazin-3-yl)oxy)-*N*′-(4-methoxybenzylidene)acetohydrazide 4. A mixture of compound **3** (0.5 g, 1.7 mmol) and anisaldehyde (0.19 mL, 1.6 mmol) in dioxane (15 mL) was refluxed for 12 h. The obtained mixture was concentrated. The precipitated solid was separated, dried fully, and recrystallized from ethanol/dioxane to give **4** as white crystals; mp: 222–224°C, yield: 45%. IR (KBr, υ/cm^{-1}): 3184 (NH), 2952, 2829 (CH_2, CH_3), 1695 (C═O), 1654 (C═N). ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm): 11.59 (s, 1H, NH, exchangeable with D_2O), 8.17–8.13, 8.12–8.08 (two d, 1H, Ar—H, H_b, J = 9.9 Hz), 7.98 (s, 1H, N═CH_e), 7.93–7.92, 7.90–7.89 (two d, 1H, Ar—H, $H_{c'}$, J_m = 1.8 Hz), 7.81, 7.75–7.72 (two d, d, 1H, Ar—H, H_c, J_o = 8.7 Hz, J_m = 1.8 Hz), 7.68–7.65 (d, 2H, Ar—H, H_g, J = 6.9 Hz), 7.53–7.50, 7.48 (two d, 1H, Ar—H, H_d, J = 8.4 Hz), 7.12–7.09 (d, 1H, Ar—H, H_a, J = 9.6 Hz), 7.01–6.98 (d, 2H, Ar—H, H_f, J = 6.6 Hz), 5.28 (s, 2H, OCH_2), 3.80 (s, 3H, OCH_3), 2.39–2.37 (two s, 3H, CH_3). MS *m/z* (%): 410 (M^+; 21.95), 260 (4.66), 232 (32.48), 177 (7.42), 162 (15.11), 150 (24.54), 76 (89.43). *Anal*. Calcd for $C_{21}H_{19}N_4O_3Cl$ (410.11): C, 61.39; H, 4.66; N, 13.64; Cl, 18.63. Found: C, 61.47; H, 4.57; N, 13.72; Cl, 18.53.

2-((6-(4-Chloro-3-methylphenyl)pyridazin-3-yl)oxy)-*N*′-(2-oxoindolin-3-ylidene)acetohydrazide 5. A mixture of compound **3** (0.5 g, 1.7 mmol) and isatin (0.25 g, 1.7 mmol) in dioxane (15 mL) was refluxed for 9 h. After evaporation of the excess solvent, the deposited solid was separated, dried fully, and recrystallized from dioxane/DMF to give **5** as yellow crystals (as lactam–lactim tautomer); mp: 178–180°C, yield: 40%. IR (KBr, υ/cm^{-1}): 3290, 3233 (NH), 2952, 2917, 2850 (CH_3, CH_2), 1727 (C═$O_{indolinone}$), 1696 (C═O_{amide}), 1655 (C═N). ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm): 13.18 (br. s, 1H, OH, exchangeable with D_2O for lactim tautomer), 12.68 (s, 1H, NH, exchangeable with D_2O for lactam tautomer), 11.26 (s, 1H, $NH_{(indolinone)}$, exchangeable with D_2O), 8.15–8.11 (d, 1H, Ar—H, H_b, J = 9.9 Hz), 7.91–7.90 (d, 1H, Ar—H, $H_{c'}$, J_m = 1.8 Hz), 7.76–7.73 (d, d, 1H, Ar—H, H_c, J_o = 8.4 Hz, J_m = 2.1 Hz), 7.58–7.54 (t, 1H, Ar—H, H_f, J = 6.9 Hz, J = 6.9 Hz), 7.54–7.51 (d, 1H, Ar—H, H_d, J = 8.4 Hz), 7.41–7.36 (t, 1H, Ar—H, H_g, J = 7.8 Hz, J = 7.2 Hz), 7.16–7.13 (d, 1H, Ar—H, H_a, J = 9.9 Hz), 7.10–7.07 (d, 1H, Ar—H, H_h, J = 7.8 Hz), 6.96–6.94 (d, 1H, Ar—H, H_e, J = 7.5 Hz), 5.46 (s, 2H, OCH_2 for lactam tautomer), 5.11 (s, 2H, OCH_2 for lactim tautomer), 2.39, 2.37 (two s, 3H, CH_3). MS *m/z* (%): 421 (M^+; 13.79), 395 (19.30), 380 (74.74), 303 (63.75), 260 (44.92), 245 (34.47), 210 (21.76), 170 (13.86). *Anal*. Calcd for $C_{21}H_{16}N_5O_3Cl$ (421.09): C, 59.79; H, 3.82; N, 16.60; Cl, 8.40. Found: C, 59.88; H, 3.86; N, 16.48; Cl, 8.31.

2-((6-(4-Chloro-3-methylphenyl)pyridazin-3-yl)oxy)-*N*′-cyclohexylideneacetohydrazide 6. A mixture of compound **3** (0.5 g, 1.7 mmol) and cyclohexanone (0.2 mL, 1.7 mmol) in dioxane/DMF (20 mL) was refluxed for 14 h. The obtained mixture was concentrated and then poured into water. The precipitated solid was separated, dried fully,

and recrystallized from ethanol to give **6** as white crystals (as lactam–lactim tautomer); mp: 223–225°C, yield: 40%. IR (KBr, υ/cm^{-1}): 3214 (NH), 2931, 2854 (CH_3, CH_2), 1669 (C═O), 1591 (C═N). ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm): 9.08 (s, 1H, NH, exchangeable with D_2O), 7.80 (d, 1H, Ar─H, $H_{c'}$, J = 1.5 Hz), 7.68–7.65 (d, 1H, Ar─H, H_b, J = 9.9 Hz), 7.52–7.50 (d, 1H, Ar─H, H_c, J = 5.7 Hz), 7.40–7.37 (d, 1H, Ar─H, H_d, J = 8.1 Hz), 7.06–7.03 (d, 1H, Ar─H, H_a, J = 9.9 Hz), 5.36 (s, 2H, OCH_2), 2.40 (s, 3H, CH_3), 2.36–2.24 (m, 4H, N═C $(CH_2)_{2(cyclohexylidene)}$), 1.71–1.62 (m, 6H, $3CH_{2(cyclohexylidene)}$). MS *m/z* (%): 372 (M$^+$; 38.86), 289 (16.50), 276 (31.94), 261 (55.19), 232 (45.02), 205 (5.30), 177 (3.82), 128 (7.14), 76 (73.69), 69 (86.65), 62 (100), 54 (87). *Anal*. Calcd for $C_{19}H_{21}N_4O_2Cl$ (372.14): C, 61.21; H, 5.68; N, 15.03; Cl, 19.51. Found: C, 61.11; H, 5.81; N, 15.11; Cl, 19.43.

2-((6-(4-Chloro-3-methylphenyl)pyridazin-3-yl)oxy)-1-(3,5-dimethyl-1*H*-pyrazol-1-yl)ethan-1-one 7 [28]. A mixture of compound **3** (0.5 g, 1.7 mmol) and acetyl acetone (0.17 mL, 1.7 mmol) in dioxane (15 mL) was refluxed for 12 h. The obtained mixture gave oil after concentration. This oil was solidified by washing with methanol. The precipitated solid was separated, dried fully, and recrystallized from diethyl ether to give **7** as yellow crystals; mp: 158–160°C, (Lit. mp: 164–166°C) [28], yield: 20%. IR (KBr, υ/cm^{-1}): 2921, 2852 (CH_2, CH_3), 1657 (C═O), 1591 (C═N). ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm): 8.10–8.07 (d, 1H, Ar─H, H_b, J = 9.9 Hz), 7.88–7.87 (d, 1H, Ar─H, $H_{c'}$, J_m = 2.1 Hz), 7.76–7.73 (d, d, 1H, Ar─H, H_c), 7.53–7.50 (d, 1H, Ar─H, H_d, J = 8.4 Hz), 7.10–7.06 (d, 1H, Ar─H, H_a, J = 9.6 Hz), 6.45 (s, 1H, C═$CH_{(pyrazole)}$), 5.12–5.07 (two d, 2H, OCH_eH_f, J = 12.9 Hz, J = 13.2 Hz), 2.39 (s, 3H, CH_3), 2.01 (s, 3H, $CH_{3a(pyrazole)}$), 1.74 (s, 3H, $CH_{3a'(pyrazole)}$). MS *m/z* (%): 356 (M$^+$; 30.50), 263 (47.01), 260 (53.07), 233 (50.39), 204 (20.07), 163 (47.82), 127 (31.95), 100 (28.33), 75 (31.69), 69 (82.48), 55 (56.91). *Anal*. Calcd for $C_{18}H_{17}N_4O_2Cl$ (356.10): C, 60.59; H, 4.80; N, 15.70; Cl, 19.94. Found: C, 60.73; H, 4.89; N, 15.64; Cl, 19.86.

2-((6-(4-Chloro-3-methylphenyl)pyridazin-3-yl)oxy)-*N*′-(2-((6-(4-chloro-3-methylphenyl)pyridazin-3-yl)oxy)acetyl) acetohydrazide 10. A mixture of compound **3** (0.5 g, 1.7 mmol) and ethyl acetoacetate (0.21 mL, 1.6 mmol) in pyridine (10 mL) was heated under reflux for 3 h. The deposited solid was separated on hot, dried, and recrystallized from DMF to give **10** as white crystals; mp: >300°C, yield: 30%. IR (KBr, υ/cm^{-1}): 3156 (NH), 2960 (CH_2, CH_3), 1675 (C═O), 1627 (C═N). ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm): 10.35 (br. s, 2H, 2OH, exchangeable with D_2O for lactim tautomer), 9.68 (br. s, 2H, 2NH, exchangeable with D_2O for lactam tautomer), 8.11–8.05 (two d, 2H, Ar─H, $2H_b$, J = 9.6 Hz), 7.87–7.86 (d, 2H, Ar─H, $2H_{c'}$, J_m = 1.8 Hz), 7.77–7.69 (two d, d, 2H, Ar─H, $2H_c$, J_o = 8.4 Hz, J_m = 1.8 Hz), 7.51–7.43 (two d, 2H, Ar─H, $2H_d$, J = 8.4 Hz, J = 8.1 Hz), 7.113–7.057 (two d, 2H, Ar─H, $2H_a$, J = 9.9 Hz), 4.90 (s, 4H, $2OCH_2$ for lactam tautomer), 4.84 (s, 4H, $2OCH_2$ for lactim tautomer), 2.36, 2.31 (two s, 6H, $2CH_3$). MS *m/z* (%): 277 (M-$C_{13}H_{10}N_3O_2Cl$; 13.99), 276 (17.21), 262 (16.61), 186 (48.33), 156 (78.89), 68 (100). *Anal*. Calcd for $C_{26}H_{22}N_6O_4Cl_2$ (552.11): C, 56.43; H, 4.01; N, 15.19; Cl, 12.81. Found: C, 56.36; H, 3.97; N, 15.27; Cl, 12.87.

5-Amino-1-(2-((6-(4-chloro-3-methylphenyl)pyridazin-3-yl) oxy)acetyl)-1′,3′-diphenyl-2,3-dihydro-1*H*,1′*H*-[3,4′-bipyrazole]-4-carbonitrile 11. A mixture of compound **3** (0.5 g, 1.7 mmol) and 2-((1,3-diphenyl-1*H*-pyrazol-4-yl) methylene)malononitrile (0.5 g, 1.7 mmol) in dioxane (15 mL) containing 1 mL of piperidine as a base was refluxed for 8 h. The obtained mixture was concentrated and then acidified with cold dilute HCl and the precipitated solid was separated, dried fully, and recrystallized from benzene to give **11** as yellow crystals (as keto-enol tautomer); mp: 198–200°C, yield: 25%. IR (KBr, υ/cm^{-1}): 3332, 3198 (NH, NH_2), 2923, 2852 (CH_2, CH_3), 2200 (C≡N) 1692 (C═O), 1664 (C═N). ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm): 11.61 (br. s, 1H, OH, exchangeable with D_2O for enol tautomer), 9.04 (s, 1H, C_5-$H_{(pyrazole)}$), 8.66 (s, 1H, NH, exchangeable with D_2O), 8.16–7.35 (m, 14H, Ar─H), 7.13–7.09 (d, 1H, Ar─H, H_a, J = 9.9), 5.23 (s, 1H, C═CH for enol tautomer), 5.02 (s, 1H, H_f), 4.87 (s, 2H, OCH_2 for keto tautomer), 3.44 (br. s, 2H, NH_2, exchangeable with D_2O), 2.39 (s, 3H, CH_3). MS *m/z* (%): 586 (M$^+$-2; 3.46), 551 (14.53), 521 (20.73), 449 (17.95), 261 (55.84), 233 (16.24), 162 (8.48), 77 (89.06), 68 (100), 56 (14.69), 62 (100). *Anal*. Calcd for $C_{32}H_{25}N_8O_2Cl$ (588.18): C, 65.25; H, 4.28; N, 19.02; Cl, 6.02. Found: C, 65.13; H, 4.21; N, 19.00; Cl, 5.92.

2-((6-(4-Chloro-3-methylphenyl)pyridazin-3-yl)oxy)-*N*-(1,3-dioxoisoindolin-2-yl)acetamide 12. A mixture of compound **3** (0.5 g, 1.7 mmol) and phthalic anhydride (0.25 g, 1.7 mmol) in n-butanol (10 mL) was heated under reflux for 8 h. The deposited solid was separated on hot, washed with ethanol, dried fully, and recrystallized from dioxane to give **12** as white crystals; mp: >300°C, yield: 20%. IR (KBr, υ/cm^{-1}): 3159 (NH), 2949 (CH_2, CH_3), 1790, 1736, 1708 (C═O_{imide}), 1656 (C═O_{amide}). ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm): 11.10 (s, 1H, NH, exchangeable with D_2O), 8.09–8.06 (d, 1H, Ar─H, H_b, J = 9.9 Hz), 7.98–7.92 (m, 5H, Ar─H), 7.88–7.87 (d, 1H, Ar─H, $H_{c'}$, J_m = 1.8 Hz), 7.76–7.73 (d, d, 1H, Ar─H, H_c, J_o = 8.7 Hz, J_m = 1.8 Hz), 7.54–7.51 (d, 1H, Ar─H, H_d, J = 8.1 Hz), 7.13–7.09 (d, 1H, Ar─H, H_a, J = 9.6 Hz), 5.07 (s, 2H, OCH_2), 2.41 (s, 3H, CH_3). MS *m/z* (%): 422 (M$^+$; 29.61), 320 (40.78), 280 (35.43), 276 (77.07). *Anal*. Calcd for $C_{21}H_{15}N_4O_4Cl$ (422.08): C, 59.65; H, 3.58; N, 13.25; Cl, 8.38. Found: C, 59.60; H, 3.56; N, 13.29; Cl, 8.32.

2-Chloro-*N*′-(2-((6-(4-chloro-3-methylphenyl)pyridazin-3-yl)oxy)acetyl)acetohydrazide 13. To a cold stirred solution of compound **3** (0.5 g, 1.7 mmol) in DMF (10 mL), chloroacetyl chloride (0.13 mL, 1.6 mmol) was added dropwise, and the obtained mixture was stirred at room temperature for 4 h and then left overnight and poured into water. The precipitated solid was separated, dried fully, and recrystallized from ethanol/dioxane to give **13** as white crystals; mp: 258–260°C, yield: 20%. IR (KBr, υ/cm^{-1}): 3176 (NH), 1678, 1658 (C═O), 1614 (C═N), 2920, 2853 (CH_2, CH_3). ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm): 10.41 (s, 2H, 2NH, exchangeable with D_2O), 8.09–8.06 (d, 1H, Ar—H, H_b, J = 9.9 Hz), 7.89 (d, 1H, Ar—H, $H_{c'}$, J_m = 1.8 Hz), 7.75–7.72 (d, d, 1H, Ar—H, H_c, J_o = 8.7 Hz, J_m = 1.8 Hz), 7.53–7.51 (d, 1H, Ar—H, H_d, J = 8.4 Hz), 7.11–7.07 (d, 1H, Ar—H, H_a, J = 9.6 Hz), 4.85 (s, 2H, OCH_2), 4.12 (s, 2H, CH_2Cl), 2.40 (s, 3H, CH_3). MS *m/z* (%): 368 (M^+; 21.76), 331 (86.69), 302 (73.50), 296 (27.70), 277 (77.40), 260 (26.32), 234 (69.81). *Anal*. Calcd for $C_{15}H_{14}N_4O_3Cl_2$ (368.04): C, 48.80; H, 3.82; N, 15.18; Cl, 19.20. Found: C, 48.81; H, 3.87; N, 15.13; Cl, 19.27.

2-((6-(4-Chloro-3-methylphenyl)pyridazin-3-yl)oxy)acetic acid 14. A solution of compound **3** (0.5 g, 1.7 mmol) in formic acid (10 mL) was heated under reflux for 12 h. The obtained mixture was concentrated. The deposited solid was separated, washed well with diethyl ether, dried fully, and recrystallized from ethanol to give **14** as white crystals; mp: 218–220°C, yield: 55%. IR (KBr, υ/cm^{-1}): 3435–2485 (br.) (OH), 2992, 2920, 2836 (CH_2, CH_3), 1711 ($C{=}O_{acid}$), 1649 (C═N), 1629 (C═C). ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm): 13.13 (br. s, 1H, OH, exchangeable with D_2O), 8.14–8.08 (two d, 1H, Ar—H, H_b, J = 9.9 Hz), 7.90, 7.89–7.88 (two d, 1H, Ar—H, $H_{c'}$, J_m = 2.1 Hz), 7.71 (two d, d, 1H, Ar—H, H_c, J_o = 8.4 Hz, J_m = 2.1 Hz), 7.54–7.51, 7.53 (two d, 1H, Ar—H, H_d, J = 8.1 Hz), 7.12–7.08 (two d, 1H, Ar—H, H_a, J = 9.9 Hz), 4.84 (s, 2H, OCH_2), 2.39, 2.37 (s, 3H, CH_3). MS *m/z* (%): 278 (M^+; 18.53), 234 (48.96), 233 (25.91), 208 (32.18), 206 (90.93), 190 (2.77), 163 (100), 128 (57.85) 127 (48.70), 102 (24.15), 77 (36.27), 63 (50.58). *Anal*. Calcd for $C_{13}H_{11}N_2O_3Cl$ (278.05): C, 56.03; H, 3.96; N, 10.05; Cl, 12.72. Found: C, 56.07; H, 3.98; N, 10.01; Cl, 12.68.

2-((6-(4-Chloro-3-methylphenyl)pyridazin-3-yl)oxy)-*N*′-(5-(((6-(4-chloro-3-methylphenyl)pyridazin-3-yl)oxy)methyl)-1,3,4-oxadiazol-2-yl)acetohydrazide 16. A solution of compound **6** (0.5 g, 1.7 mmol) in triethyl orthoformate (20 mL) was refluxed for 72 h. The obtained mixture was concentrated. The precipitated solid was separated, washed well with ethanol, dried fully, and recrystallized from dioxane to give **14** as white crystals; mp: 208–210°C, yield: 35%. IR (KBr, υ/cm^{-1}): 3224 (NH), 1656 (C═O), 1633 (C═N). ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm): 10.03 (s, 1H, NH, exchangeable with D_2O), 8.38 (br. s, 1H, NH, exchangeable with D_2O), 8.10–7.48 (m, 8H, Ar—H), 7.08–7.05 (d, 2H, Ar—H, $2H_a$, J = 9.9 Hz), 5.09–4.78 (m, 4H, $2OCH_2$), 2.37, 2.27 (2 s, 6H, $2CH_3$). MS *m/z* (%): 590 (M^+-2; 2.29), 578 (9.44), 302 (100), 261 (15.81), 233 (8.95), 204 (9.13), 192 (12.29), 163 (8.03) 150 (10.36), 126 (20.48), 68 (33.47), 57 (43.01), 44 (78.74). *Anal*. Calcd for $C_{27}H_{22}N_8O_4Cl_2$ (592.11): C, 54.65; H, 3.74; N, 18.88; Cl, 11.95. Found: C, 54.71; H, 3.77; N, 18.83; Cl, 11.89.

2-(2-((6-(4-Chloro-3-methylphenyl)pyridazin-3-yl)oxy)acetyl)-*N*-phenylhydrazine-1-carbothioamide 17. A mixture of compound **3** (0.5 g, 1.7 mmol) and phenyl isothiocyanate (0.3 mL, 1.7 mmol) in DMF (15 mL) was refluxed for 20 h. The precipitated solid on hot was separated, washed well with diethyl ether, and dried fully to give **17** as white crystals; mp: 248–250°C, yield: 20%. IR (KBr, υ/cm^{-1}): 3158 (NH), 2959, 2835 (CH_2, CH_3), 1671 (C═O), 1628 (C═N), 1163 (C═S). ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm): 10.60 (s, NHPh, exchangeable with D_2O), 10.36 (s, 1H, NHCS, exchangeable with D_2O), 9.65 (s, 1H, CONH, exchangeable with D_2O), 8.08–8.05 (d, 1H, Ar—H, H_b, J = 9.3 Hz), 7.93 (s, 1H, Ar—H, $H_{c'}$), 7.72–7.69 (d, 1H, Ar—H, H_c, J_o = 8.7 Hz), 7.61–7.33 (m, 6H, Ar—H, Ph + H_d), 7.09–7.06 (d, 1H, Ar—H, H_a, J = 9.6 Hz), 4.84 (s, 2H, OCH_2), 2.36 (s, 3H, CH_3). MS *m/z* (%): 427 (M^+; 23.60), 426 (27.34), 290 (30.09), 261 (23.55), 260 (27.76), 235 (100), 178 (45.55), 151 (31.46), 143 (27.53), 101 (24.49), 76 (72.84), 63 (92.80), 50 (81.12). *Anal*. Calcd for $C_{20}H_{18}N_5O_2ClS$ (427.09): C, 56.14; H, 4.24; N, 16.37; Cl, 8.28; S, 7.49. Found: C, 56.21; H, 4.29; N, 16.39; Cl, 8.22; S, 7.56.

5-(((6-(4-Chloro-3-methylphenyl)pyridazin-3-yl)oxy)methyl)-1,3,4-oxadiazole-2-thiol 18. A mixture of compound **3** (0.5 g, 1.7 mmol) and carbon disulfide (5 mL) in dioxane (10 mL) in the presence of aqueous potassium hydroxide solution (0.5 g/10 mL) was refluxed on a water bath for 24 h. The obtained mixture was acidified with cold dilute hydrochloric acid and the precipitated solid was separated and then later poured on Na_2CO_3 solution, followed by filtration. The filtrate was acidified by dilute HCl to get **14**. While the residue obtained was separated, dried well, and recrystallized from ethanol to give **18** as orange crystals; mp: 192–194°C, yield: 30%. IR (KBr, υ/cm^{-1}): 2918, 2849 (CH_2, CH_3), 1648 (C═N), 1630 (C═C). ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm): 13.19 (br. s, SH, exchangeable with D_2O), 8.10–8.07 (d, 1H, Ar—H, H_b, J = 9.9 Hz), 7.92 (s, 1H, Ar—H, $H_{c'}$), 7.73–7.70 (d, 1H, Ar—H, H_c, J_o = 8.4 Hz), 7.53–7.50 (d, 1H, Ar—H, H_d, J = 8.4 Hz), 7.12–7.08 (d, 1H, Ar—H, H_a, J = 9.9 Hz), 4.84 (s, 2H, OCH_2), 2.39 (s, 3H, CH_3). MS *m/z* (%): 334 (M^+; 9.70), 304 (11.55), 260 (17.95), 234 (54.31), 163 (76.87), 162

(84.35), 128 (45.40), 127 (47.73), 115 (43.70), 102 (16.66), 77 (76.15), 64 (96.12), 63 (80.40), 40 (100). *Anal*. Calcd for $C_{14}H_{11}N_4O_2ClS$ (334.03): C, 50.23; H, 3.31; N, 16.74; Cl, 10.59; S, 9.58. Found: C, 50.19; H, 3.29; N, 16.75; Cl, 10.61; S, 9.54.

5-(((6-(4-Chloro-3-methylphenyl)pyridazin-3-yl)oxy) methyl)-2,4-dihydro-3*H*-1,2,4-triazole-3-thione 19. A mixture of compound **3** (0.5 g, 1.7 mmol), ammonium isothiocyanate (0.13 g, 1.7 mmol) in the presence of concentrated hydrochloric acid (1.4 mL), and water (8.5 mL) was heated under reflux for 30 min. The precipitated solid on hot was separated, washed well with water, dried fully, and recrystallized from ethanol/dioxane to give **19** as white crystals; mp: 288–290°C, yield: 25%. IR (KBr, υ/cm^{-1}): 3170 (NH), 2952, 2920 (CH_2, CH_3), 1649 (C═N), 1629 (C═C), 1244 (C═S). ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm): 10.34 (s, NH, exchangeable with D_2O), 1028 (s, NH, exchangeable with D_2O), 8.11–8.08 (d, 1H, Ar─H, H_b, J = 9.3 Hz), 7.887–7.880 (d, 1H, Ar─H, $H_{c'}$, J_m = 2.1 Hz), 7.74–7.70 (d, d, 1H, Ar─H, H_c, J_o = 8.4 Hz, J_m = 2.4 Hz), 7.53–7.51 (d, 1H, Ar─H, H_d, J = 8.4 Hz), 7.12–7.09 (d, 1H, Ar─H, H_a, J = 9.6 Hz), 4.84 (s, 2H, OCH_2), 2.39 (s, 3H, CH_3). MS *m/z* (%): 333 (M^+; 16.89), 307 (20.34), 274 (7.66), 260 (12.62), 233 (18.58), 207 (21.79), 205 (32.83), 190 (14.91), 169 (48.64), 127 (77.31), 115 (55.56), 80 (100), 77 (55.10), 63 (61.16), 45 (74.31). *Anal*. Calcd for $C_{14}H_{12}N_5OClS$ (333.79): C, 50.38; H, 3.62; N, 20.98; Cl, 10.62; S, 9.60. Found: C, 50.49; H, 3.67; N, 20.93; Cl, 10.71; S, 9.54.

IN VITRO ANTITUMOR EVALUATION

MTT assay. MTT assay employed for antitumor screening of pyridazine derivatives **3**–**19** toward hepatocellular carcinoma (HepG2) and mammary gland (MCF-7) as two cell lines was from ATCC *via* Holding company for biological products and vaccines (VACSERA), Cairo, Egypt. The reagents utilized were RPMI-1640 medium, MTT, DMSO (Sigma Co., St. Louis, USA), and fetal bovine serum (GIBCO, UK). Doxorubicin was utilized as the reference anticancer drug.

The two cell lines aforementioned were utilized to detect the inhibitory effects of compounds on cell growth utilizing the MTT assay [30,31]. This colorimetric test is depending on the conversion of the yellow tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase in viable cells to a purple formazan derivative. In RPMI-1640 medium with 10% fetal bovine serum, the cells were cultured. Antibiotics added were 100 units/mL penicillin and 100 μg/mL streptomycin at 37°C in a 5% CO_2 incubator. Further, in a 96-well plate at a density of (1.0×10^4 cells/well) at 37°C for 48 h under 5% CO_2, the cells were seeded. After incubation, the cells were treated with variant concentrations of compounds and put in the incubator for 24 h and then 20 μL of MTT solution at 5 mg/mL was added and incubated for 4 h. Into each well, DMSO (100 μL) was added to dissolve the purple formazan formed. At 570 nm absorbance, the colorimetric assay was measured and recorded by utilizing a plate reader (EXL 800, USA).

Calculation of the relative cell viability (%) = (*A* of treated samples/*A* of untreated sample) × 100.

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