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Synthesis and evaluation of novel 4-chloro-5-(cyclic/acyclic amino)-2-*p*-tolyl-(2*H*)-pyridazin-3-one derivatives as anticancer, antiangiogenic, and antioxidant agents

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Abstract: Pyridazinone is a unique template that is associated with several pharmacological activities. A new class of pyridazinone derivatives containing cyclic/acyclic amine moiety was synthesized and evaluated for their *in vitro* cytotoxic activity against HEP3BPN 11 (liver), MDA 453 (breast) and HL60 (leukemia) cancer cells. Three derivatives **4a** (76.78±0.7 against HEP3BPN 11), **4b** (71.55±0.88 against HL60), and **4c** (79.04±0.2 against HEP3BPN 11 & 73.09±0.1 against MDA 453) showed promising anticancer activity were further tested for their potential to inhibit the proangiogenic cytokines involved in tumor progression. Free radical scavenging activities of these compounds were demonstrated using 2,2-diphenyl-1-picryl hydrazine (DPPH), OH and superoxide anion radicals.

Keywords: Synthesis, Pyridazinones, Anticancer, Antiangiogenic, Antioxidant agents.

Introduction

Besides the invention of plethora of cancer chemotherapeutic agents, drugs having more therapeutic index and safe on health grounds are appreciated in the ‘target rich lead poor’ scenario of anticancer research [1]. The currently used chemotherapeutic agents do not distinctively

target tumor cells, but rather obstruct with cell division or hamper enzymes involved in DNA replication or metabolism. These drugs hence moreover damage the normal dividing cells of rapidly regenerating tissues, like those of the bone marrow, gut mucosa and hair follicles. Cancer chemotherapy is restricted by a lack of specificity, results into the damage to cancer

cells as well as normal cells. This generates a narrow therapeutic index [2-3]. Trying to evade the side effects appearing as a result of toxicities to normal tissues, we frequently give suboptimal doses of cancer chemotherapeutic agents. The result is often incomplete tumor response, eventual failure of therapy, early disease relapse, drug resistance, and metastatic disease [4].

Angiogenesis is defined as process of development of new blood vessels from the pre-existing vessels. This physiological process is governed by numerous pro- and anti-angiogenic factors, like vascular endothelial growth factor (VEGF), basic fibroblast growth factor (FGFb), epidermal growth factor (EGF), and transforming growth factor-beta (TGF- β) [5]. The mechanism of action of every of these factors is diverse, as are their origin and the stimuli for their production. The angiogenic switch refers to the balance amongst pro- and anti-angiogenic factors. Consequently, profiling of these factors is vital to understand angiogenesis. The growth of tumors is dependent on their ability to activate angiogenesis, to induce the growth of blood vessels to provide them with oxygen and nutrients. In view of the side effects linked with traditional chemotherapies and the probability of hindering a tumor's supply of oxygen and nutrient, there has been huge attention taken in the targeting of tumor vasculature and great effort has been focused towards the improvement of anti-angiogenic agents that could interrupt this angiogenesis. Stabilization of tumor vasculature by anti-angiogenic drugs is a rising model for improving the efficiency of cytotoxic chemotherapy [6-9].

Free radicals are the highly reactive molecules which carries the unpaired electrons. Free radicals lead to the causation of oxidative stress. Oxidative stress is an imbalance between oxidants and antioxidants in favor of the enormous production of free radicals

potentially induces cellular damage [10]. Oxidative stress can damage lipids, proteins, enzymes, carbohydrates and DNA in cells and tissues, which results into membrane damage, fragmentation of molecules like DNA, enzymes and structural proteins and even leading to cell death induced by DNA fragmentation and lipid peroxidation [11]. These adverse effects of oxidative stress establish the molecular origin in the development of numerous diseases as well as cancer. Consequently cancer chemotherapy agents acquiring antioxidant potential are more acknowledged in the mainstream of pharmaceutical research.

Arylpyridazinones comprise a very interesting class of compounds because of their significant biological and pharmaceutical activities. They have been described to possess broad spectrum biological activities such as antimicrobial [12], analgesic [13], anti-inflammatory [14], antihypertensive [15], glucan synthase inhibitors [16], HIV-1 reverse transcriptase inhibitors [17], antiasthmatic [18], antisecretory and antiulcer [19], antianemic [20], antidepressant [21], antinociceptive [22], cardiogenic [23], and anticancer activities [24]. In continuation with our current interest toward synthesis of bioactive nitrogen containing compounds [25-27], in this work we explored the possibility to combine the potential anti-carcinogenic effects of pyridazinone moiety and amine scaffold. Thus, we synthesized a new class of pyridazinone derivatives with amines and evaluated their anticancer, antiangiogenic and antioxidant potential.

Results and Discussion

Chemistry

In the present work, we have synthesized the key intermediate compound [2-(methylphenyl)]-4,5-dichloropyridazin-3-one **3** by refluxing mucochloric acid **1** with 4-methylphenyl

hydrazine **2** in diluted H_2SO_4 (3M) for 2 h. In the previously reported approach [16], intermediate **3** was prepared by the reaction of mucochloric acid **1** with 4-methylphenyl hydrazine hydrochloride in aqueous NaOH at 100°C for 24 h. As compared to the reported method, our approach was superior in terms of time, yield and economical, towards the synthesis of [2-(4-methylphenyl)]-4,5-dichloropyridazin-3-one **3** and this may be regarded as efficient

approach. The title 4-chloro-5-(cyclic/acyclic amino)-2-*p*-tolyl-(2*H*)-pyridazin-3-one derivatives **4a-l** were obtained in 67-90% yields as depicted in **Table 1** by refluxing intermediate **3** with various aliphatic/cyclic saturated amines in the presence of CS_2CO_3 (cesium carbonate) in acetonitrile. We used the different aliphatic amines and studied the structure activity relationship (SAR).

Table 1: Synthesis of 4-chloro-5-(cyclic/acyclic amino)-2-*p*-tolyl-(2*H*)-pyridazin-3-one derivatives **4a-l**

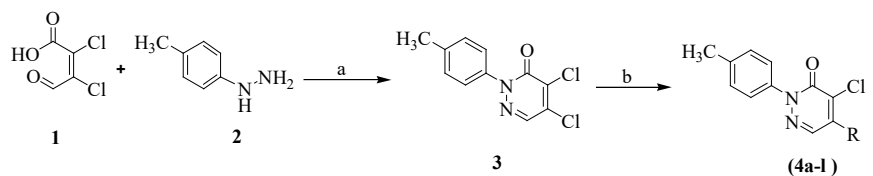
Entry	Product ^a	cyclic /acyclic amine	Time (h)	Yield ^b (%)
4a			2.30	70
4b			2	85
4c			2	67
4d			2	70
4e			1.30	85
4f			2.30	84

continue

Entry	Product ^a	cyclic /acyclic amine	Time (h)	Yield ^b (%)
4g			1.30	68
4h			1.30	75
4i			1.30	90
4j			2	80
4k			2	70
4l			2	90

^a All the products were characterized by ¹H NMR, LCMS, IR and ¹³C NMR.

^b Isolated yields.



R = Aliphatic amine / cyclised saturated amine

Reagent and reaction conditions:

a) Diluted H₂SO₄, 2 h reflux.

b) Aliphatic amine / cyclised saturated amine, CS₂CO₃, acetonitrile 1.5 - 2.5 h reflux.

Scheme 1: Synthesis of novel 4-chloro-(5-cyclic/acyclic amino)-2-*p*-tolyl-2H-pyridazin-3-ones

Biology

Cytotoxicity assay

The synthesized compounds (**4a-l**) were tested for *in vitro* biological screening for their cytotoxicity potential towards the cancer cell lines using MTT assay. The cytotoxicity studies were determined against three human cancer cell lines, HEP3BPN 11 (liver), MDA 453 (breast) and HL 60 (leukemia) and the results are presented in **Table 2**. This table indicates the percentage cytotoxic activity (dose dependent) of the synthesized compounds at 100 μ M concentration. All these compounds possess common 4-chloro-2-*p*-tolyl-2*H*-pyridazin-3-one nucleus as A-ring. The substitutions at C-5 position of the A-ring play an important role in determining the potency of the compounds **4a-l**. With respect to HEP3BPN 11 cell line, compounds **4a** (76.78 ± 0.1) and **4e** (79.04 ± 0.2) found to have a good activity when compared with standard (84.46 ± 0.73). The compound **4e** (73.09 ± 0.10) indicates the highest cytotoxicity against MDA 453 cell line. Also, compound **4g** (76.33 ± 0.10) have a high cytotoxic activity against HL 60 cell line. The SAR study of aminopyridazinone derivatives **4a-g** with respect to HEP3BPN 11 cell line revealed that compounds containing piperidine **4a** and cyclopentylamine **4e** at C-5 position of A-ring have higher cytotoxicity than substituted piperazines **4b-d**, **4l**, homo piperazines **4k**, pyrrolidine **4f**, N, N-dimethyl diamine **4j** and N, N-dimethyl ethylene diamine **4g**. In case of MDA 453, compound containing cyclopentylamine **4e** at C-5 position of A-ring exhibited higher cytotoxicity than rest of the compounds. As far as HL 60 is concerned, compound containing N, N-dimethyl ethylene diamine **4g** at C-5 position of A-ring showed better activity when compared to remaining compounds.

Entry	Compound	(%) Cytotoxicity		
		HEP3BPN11	MDA 453	HL 60
1	4a	*76.78 $\pm$ 0.1	61.06 $\pm$ 0.97	*69.54 $\pm$ 0.3
2	4b	*68.67 $\pm$ 0.98	*67.98 $\pm$ 0.9	*71.55 $\pm$ 0.88
3	4c	*68.56 $\pm$ 0.46	63.70 $\pm$ 0.54	*68.87 $\pm$ 0.25
4	4d	*71.22 $\pm$ 0.9	NP	*67.03 $\pm$ 0.15
5	4e	*79.04 $\pm$ 0.2	*73.09 $\pm$ 0.10	62.72 $\pm$ 0.05
6	4f	*69.04 $\pm$ 0.25	60.34 $\pm$ 0.12	65.01 $\pm$ 0.31
7	4g	64.33 $\pm$ 0.07	60.96 $\pm$ 0.05	*76.33 $\pm$ 0.10
8	4h	60.64 $\pm$ 0.23	55.73 $\pm$ 0.04	*70.77 $\pm$ 0.55
9	4i	*69.45 $\pm$ 0.44	*71.79 $\pm$ 0.4	64.08 $\pm$ 0.09
10	4j	55.04 $\pm$ 0.26	50.09 $\pm$ 0.58	60.72 $\pm$ 0.18
11	4k	60.04 $\pm$ 0.34	*69.34 $\pm$ 0.7	*70.01 $\pm$ 0.08
12	4l	NP	*69.87 $\pm$ 0.3	*69.37 $\pm$ 0.5
13	M	84.46 $\pm$ 0.73	88.73 $\pm$ 0.87	82.77 $\pm$ 0.01

Table 2 Cytotoxicity by MTT assay at 0.01 mM (**4a-l**), NP- % Cytotoxicity was less than 10%

M- Methothrexate, Results are expressed as the mean values from three independent experiments $\pm$ Standard Deviation (SD). *P $\leq$ 0.05 as compared to control.

Antiangiogenic activity

The compounds **4a**, **4e** and **4g** showing better cytotoxicity against chosen cancer cell lines were further assessed for inhibition of selected cytokines like TNF α , IGF1, VEGF, IL6, FGFb, TGF β , EGF and Leptin. These cytokines are depicted to be associated in advancement of tumor growth principally by selecting enormous vasculature for the promotion of tumor growth [28]. The results are summarized in Table 3. Compound **4a** is found to be potent antiangiogenic agent against VEGF and Leptin. Compound **4e** has shown potent TNF α , VEGF, FGFb, TGF β , and EGF inhibition, while **4g** has shown to inhibit TNF α , VEGF, IL6, and EGF. Thus, antiangiogenic study of compounds **4a**, **4e** and **4g** clearly demonstrates their involvement in inhibition process of angiogenesis by inhibiting the proangiogenic proteins like TNF α , IGF1, VEGF, IL6, FGFb, TGF β , EGF and Leptin.

Sample	Inhibition of tumor growth promoting cytokine (%)							
	TNF α	IGF1	VEGF	IL6	FGFb	TGFb	EGF	Leptin
4a	74.22 ± 0.08	58.87 ± 0.06	*87.56 ± 0.07	71.36 ± 0.09	*75.37 ± 0.18	*76.27 ± 0.10	64.56 ± 0.08	*78.38 ± 0.20
4c	*82.21 ± 0.21	67.72 ± 0.19	*83.69 ± 0.09	64.65 ± 0.04	*81.67 ± 0.21	*77.64 ± 0.12	*79.46 ± 0.05	69.51 ± 0.10
4g	*85.83 ± 0.05	72.58 ± 0.03	*79.37 ± 0.04	*82.56 ± 0.11	64.98 ± 0.07	61.52 ± 0.04	*81.49 ± 0.07	75.46 ± 0.07
Suramin	*89.76 ± 0.04	*87.89 ± 0.13	*93.88 ± 0.02	*89.90 ± 0.11	*90.34 ± 0.03	*91.25 ± 0.10	*86.67 ± 0.06	*85.37 ± 0.03

Table 3 Effects of selected compounds on the tumor growth promoting cytokines at 0.01 mM concentration. Results are expressed as the mean values from three independent experiments $\pm$ Standard Deviation (SD). *P $\leq$ 0.05 as compared to control.

Antioxidant activity

The DPPH radicals scavenging assay has been used for primary screening of the amino-pyridazinone derivatives for their antioxidant activity. All the results of study are depicted in **Table 4**. The present results demonstrate that, all compounds have potential to interact with the stable free radical DPPH, which specify their effective radical scavenging capability. Compound **4a** exhibited better antioxidant property (70.27%) when compared with the standard ascorbic acid (76.45%), whereas rest of the compounds showed moderate radical scavenging activity. The in general range of DPPH radical activity was found to be 56.26 to 70.27%. The OH radicals are highly reactive amongst the relevant oxygen species and that influence each kind of molecule present in living system. Significant bimolecules like phospholipids, sugar, DNA bases, amino acids, and organic acids may react with OH radicals and can alter regular physiological utilities of cells [29]. It was found that, all compounds **4a-l** demonstrated better OH radical scavenging activity (71.37 – 87.92%) when compared with the standard ascorbic acid (79.37%). The

results of SOR scavenging activities shows that, compound **4a** (81.02%) exhibited better SOR scavenging activity as compared to the standard ascorbic acid (72.58%), while rest of the compounds showed moderate SOR scavenging activity (46.26 – 69.59%).

The SAR study shows that, compound containing piperidine **4a** at C-5 position of A-ring have highest DPPH and SOR activity than compounds containing substituted piperazines **4b-d**, **4l**, homo piperazines **4k**, pyrrolidine **4f**, N, N-dimethyl diamine **4j** and N,N-dimethyl ethylene diamine **4g** at C-5 position of A-ring. It is significant to mention that compounds having saturated cyclic amines as well as aliphatic amine **4a-l** at C-5 position of A-ring displayed better OH radical scavenging activity (71.37 – 87.92%) as compared to the standard ascorbic acid (79.37%).

Table 4 % DPPH, OH, SOR activity of the synthesized compounds (4a-l).

*P $\leq$ 0.05 as compared to control.

Entry	Compound	Concentration (mM)	% DPPH Activity	% OH Activity	% SOR Activity
1	4a	0.5	52.39 $\pm$ 0.63	*75.41 $\pm$ 0.53	*67.33 $\pm$ 0.48
		0.1	*70.27 $\pm$ 0.43	*84.96 $\pm$ 0.37	*81.02 $\pm$ 0.83
2	4b	0.5	47.62 $\pm$ 0.21	68.90 $\pm$ 0.56	37.50 $\pm$ 0.50
		0.1	*67.82 $\pm$ 0.29	*87.92 $\pm$ 0.72	*63.65 $\pm$ 0.30
3	4c	0.5	42.86 $\pm$ 0.20	54.27 $\pm$ 0.77	56.55 $\pm$ 0.53
		0.1	56.26 $\pm$ 0.38	*86.60 $\pm$ 0.71	*69.59 $\pm$ 0.12
4	4d	0.5	57.15 $\pm$ 0.48	65.00 $\pm$ 0.14	43.80 $\pm$ 0.25
		0.1	*67.94 $\pm$ 0.50	*71.37 $\pm$ 0.29	*64.92 $\pm$ 0.39
5	4e	0.5	55.56 $\pm$ 0.66	*71.50 $\pm$ 0.59	38.23 $\pm$ 0.50
		0.1	*67.91 $\pm$ 0.18	*82.36 $\pm$ 0.75	59.27 $\pm$ 0.66
6	4f	0.5	49.21 $\pm$ 0.21	*78.20 $\pm$ 0.37	28.20 $\pm$ 0.72
		0.1	*67.83 $\pm$ 0.68	*86.92 $\pm$ 0.66	46.26 $\pm$ 0.48
7	4g	0.5	44.45 $\pm$ 1.11	*70.20 $\pm$ 0.88	45.32 $\pm$ 0.12
		0.1	*66.83 $\pm$ 0.20	*82.36 $\pm$ 1.15	59.57 $\pm$ 0.78
8	4h	0.5	42.86 $\pm$ 0.60	64.27 $\pm$ 0.26	46.55 $\pm$ 0.82
		0.1	56.26 $\pm$ 0.25	*80.26 $\pm$ 0.67	55.59 $\pm$ 0.38
9	4i	0.5	44.45 $\pm$ 1.17	*70.02 $\pm$ 0.18	45.32 $\pm$ 0.59
		0.1	*66.83 $\pm$ 0.09	*82.36 $\pm$ 0.26	59.57 $\pm$ 0.33
10	4j	0.5	40.56 $\pm$ 0.12	60.50 $\pm$ 0.82	38.75 $\pm$ 1.59
		0.1	52.91 $\pm$ 0.52	*75.36 $\pm$ 0.90	50.27 $\pm$ 0.14
11	4k	0.5	50.21 $\pm$ 0.90	*72.00 $\pm$ 0.39	40.20 $\pm$ 0.52
		0.1	*69.83 $\pm$ 0.32	*87.92 $\pm$ 0.22	*65.26 $\pm$ 1.31
12	4l	0.5	49.21 $\pm$ 0.48	*78.00 $\pm$ 0.66	28.20 $\pm$ 0.72
		0.1	*67.83 $\pm$ 0.51	*86.92 $\pm$ 0.66	46.26 $\pm$ 0.15
13	Ascorbic acid	0.1	76.45 $\pm$ 0.75	79.37 $\pm$ 0.47	72.58 $\pm$ 0.77

Materials and Methods

All the chemicals were purchased from Aldrich Chemical Co., USA. All the solvents were purchased from S. D. Fine chemicals. Thin layer chromatography plates (0.2 mm thickness sheet) were purchased from Merks kieselgel 60F254. IR spectra (KBr disks) were recorded using a Perkin–Elmer 237 spectrophotometer. ^1H NMR and ^{13}C NMR spectra were recorded on Varian (300 and 400 MHz) spectrometer using DMSO- d_6 and CDCl_3 as solvent, with TMS as an internal reference. Mass spectra were recorded on a Shimadzu LCMS-QP 1000 EX. TLC was performed on silica gel coated plates for monitoring the reactions.

General procedure for compounds

Synthesis of [2-(4-methylphenyl)]-4,5-dichloropyridazin-3-one (3).

To a mixture of mucochloric acid **1** (14.99 g, 88.75 mmol) in diluted sulphuric acid (3M, 150 mL), *p*-methylphenyl hydrazine **2** (10.84 g, 88.75 mmol) was added and reaction mixture was stirred at room temperature. The solution was refluxed for 2 h. After completion of reaction as indicated by TLC, the suspension was filtered, washed with water and dried under high vacuum to afford crude solid compound **3**. Yield: 96%; ^1H NMR (DMSO- d_6 , 400 MHz): δ 8.32 (s, 1H); 7.41 (d, J = 8.4 Hz, 2H); 7.32 (d, J = 8.4 Hz, 2H); 2.37 (s, 3H). LCMS: m/e 255 (M+1).

Synthesis of 4-chloro-5-(aliphatic/cyclic saturated amino)-2-p-tolyl-(2H)-pyridazin-3-one (4a-l).

To a mixture of crude [2-(4-methylphenyl)]-4,5-dichloropyridazin-3-one **3** (1.99 g; 7.84 mmol) in acetonitrile (20 mL), substituted amine (10 mmol) and anhydrous cesium carbonate (2.54 g; 7.84 mmol) was added. The reaction mixture was then refluxed for 1.5 to 2.5 h. After completion

of reaction (TLC), reaction mixture was poured over crushed ice, extracted with ethyl acetate (30 mL x 3), washed with water (30 mL x 2), brine (30 mL), dried (anhydrous Na_2SO_4) and concentrated under reduced pressure. The crude product was purified by recrystallization with petroleum ether / diethyl ether.

4-chloro-5-(piperidin-1-yl)-2-p-tolylpyridazin-3(2H)-one(4a).

Off white solid, MP: 124-125°C, ^1H NMR (DMSO- d_6 , 400 MHz): δ 8.03 (s, 1H), 7.36 (d, J = 8.0 Hz, 2H), 7.27 (d, J = 8.0 Hz, 2H), 3.44 - 3.41 (m, 4H), 2.35 (s, 3H), 1.65-1.61 (m, 6H). LCMS: m/e 304(M+1). Anal. Calcd for $\text{C}_{16}\text{H}_{18}\text{ClN}_3\text{O}$: C, 63.26; H, 5.97; N, 13.83. Found: C, 63.03; H, 5.95; N, 13.73%.

4-chloro-5-(4-methyl-1, 4-diazepan-1-yl)-2-p-tolylpyridazin-3(2H)-one(4b).

Light yellow solid, MP: 122-123°C, IR cm^{-1} (KBr): 2928, 2795, 1645, 1605, 1509, 1315. ^1H NMR (DMSO- d_6 , 400 MHz): δ 8.01 (s, 1H), 7.37 (d, J = 8.0 Hz, 2H), 7.26 (d, J = 8.0 Hz, 2H), 3.76 - 3.71 (m, 4H), 2.68 (t, J = 4.4 Hz, 2H), 2.54 (t, J = 5.6 Hz, 2H), 2.35 (s, 3H), 2.28 (s, 3H), 1.91 - 1.88 (m, 2H). ^{13}C NMR (CDCl_3 , 300 MHz): δ 158.19, 146.64, 139.01, 137.60, 130.23, 129.04, 124.84, 110.89, 58.45, 57.25, 51.05, 50.90, 46.65, 28.14, 21.01. LCMS: m/e 333 (M+1). Anal. Calcd for $\text{C}_{17}\text{H}_{21}\text{ClN}_4\text{O}$: C, 61.35; H, 6.36; N, 16.83. Found: C, 61.43; H, 6.57; N, 16.77%.

4-chloro-5-(4-ethylpiperazin-1-yl)-2-p-tolylpyridazin-3(2H)-one(4c).

Light yellow solid, MP: 124-125°C, IR cm^{-1} (KBr): 2964, 2822, 2350, 1640, 1416, 1151. ^1H NMR (DMSO- d_6 , 400 MHz): δ 8.06 (s, 1H), 7.36 (d, J = 8.0 Hz, 2H), 7.27 (d, J = 7.6 Hz, 2H), 3.47 - 3.32 (m, 8H), 2.38 - 2.35 (m, 5H), 1.03 (t, J = 7.2 Hz, 3H). ^{13}C NMR (CDCl_3 ,

400 MHz): δ 158.10, 147.17, 139.10, 138.02, 131.34, 129.27, 125.02, 117.11, 52.65, 52.28, 48.05, 21.17, 11.85. LCMS: m/e 333(M+1). Anal. Calcd for $C_{17}H_{21}ClN_4O$: C, 61.35; H, 6.36; N, 16.83. Found: C, 61.14; H, 6.05; N, 16.74%.

4-chloro-5-(4-methylpiperazin-1-yl)-2-p-tolylpyridazin-3(2H)-one(4d).

Off white solid, MP: 180-181°C, IR cm^{-1} (KBr): 2943, 2322, 1660, 1601, 1298, 1145. ^1H NMR (DMSO- d_6 , 400 MHz): δ 8.06 (s, 1H), 7.36 (d, $J = 7.6$ Hz, 2H), 7.27 (d, $J = 7.6$ Hz, 2H), 3.47 - 3.41 (m, 4H), 2.46 - 2.42 (m, 4H), 2.35 (s, 3H), 2.22 (s, 3H). ^{13}C NMR (DMSO- d_6 , 400 MHz): δ 158.10, 147.18, 138.98, 138.05, 131.36, 129.28, 125.02, 117.22, 54.88, 48.88, 46.06, 21.17. LCMS: m/e 319(M+1). Anal. Calcd for $C_{16}H_{19}ClN_4O$: C, 60.28; H, 6.01; N, 17.57. Found: C, 60.11; H, 6.17; N, 17.36%.

4-chloro-5-(cyclopentylamino)-2-p-tolylpyridazin-3(2H)-one(4e).

Off white solid, MP: 170-171°C, IR cm^{-1} (KBr): 3302, 2957, 2363, 1647, 1610, 1429. ^1H NMR (DMSO- d_6 , 400 MHz): δ 8.08 (s, 1H), 7.35 (d, $J = 7.6$ Hz, 2H), 7.25 (d, $J = 8.0$ Hz, 2H), 6.39 (d, 1H), 4.16 - 4.14 (m, 1H), 2.35 (s, 3H), 1.98 - 1.96 (m, 2H), 1.71 - 1.55 (m, 6H). LCMS: m/e 304(M+1). Anal. Calcd for $C_{16}H_{18}ClN_3O$: C, 63.26; H, 5.97; N, 13.83. Found: C, 63.43; H, 5.64; N, 13.87%.

4-chloro-5-(pyrrolidin-1-yl)-2-p-tolylpyridazin-3(2H)-one(4f).

White solid, MP: 156-157°C, ^1H NMR (DMSO- d_6 , 400 MHz): δ 7.91 (s, 1H), 7.35 (d, $J = 7.2$ Hz, 2H), 7.26 (d, $J = 6.8$ Hz, 2H), 3.74 - 3.71 (m, 4H), 2.34 (s, 3H), 1.91 - 1.87 (m, 4H). LCMS: m/e 290(M+1). Anal. Calcd for $C_{15}H_{16}ClN_3O$: C, 62.18; H, 5.57; N, 14.50. Found: C, 62.53; H, 5.26; N, 14.45%.

5-(2-(dimethylamino) ethylamino)-4-chloro-2-p-tolylpyridazin-3(2H)-one(4g).

Off white solid, MP: 115-116°C, IR cm^{-1} (KBr): 3374, 2945, 2768, 1625, 1441, 1333. ^1H NMR (DMSO- d_6 , 400 MHz): δ 8.04 (s, 1H), 7.35 (d, $J = 4.0$ Hz, 2H), 7.26 (d, $J = 4.0$ Hz, 2H), 6.51 (bs, 1H), 3.46 (m, 2H), 2.50 - 2.35 (m, 5H), 2.19 (s, 6H). ^{13}C NMR (DMSO- d_6 , 400 MHz): δ 157.35, 143.68, 138.27, 137.78, 129.25, 126.68, 125.18, 107.56, 57.38, 45.08, 40.04, 21.16. LCMS: m/e 307(M+1). Anal. Calcd for $C_{15}H_{19}ClN_4O$: C, 58.72; H, 6.24; N, 18.26. Found: C, 58.87; H, 6.03; N, 18.04%.

5-(dimethylamino) methylamino)-4-chloro-2-p-tolylpyridazin-3(2H)-one(4h).

Off white solid, MP: 110-115°C, IR cm^{-1} (KBr): 3350, 2945, 2768, 1625, 1435, 1225. ^1H NMR (DMSO- d_6 , 400 MHz): δ 8.04 (s, 1H), 7.35 (d, $J = 4.0$ Hz, 2H), 7.26 (d, $J = 4.0$ Hz, 2H), 6.51 (bs, 1H), 3.82 (s, 2H), 2.40 - 2.19 (s, 9H). ^{13}C NMR (DMSO- d_6 , 400 MHz): δ 157.45, 142.58, 137.23, 135.58, 128.35, 125.68, 122.45, 107.56, 67.38, 42.10, 21.26. LCMS: m/e 293(M+1). Anal. Calcd for $C_{14}H_{17}ClN_4O$: C, 57.44; H, 5.85; N, 19.14. Found: C, 57.82; H, 5.20; N, 19.04%.

4-chloro-5-morpholino-2-p-tolylpyridazin-3(2H)-one(4i).

Off white solid, MP: 170-171°C, ^1H NMR (DMSO- d_6 , 400 MHz): δ 8.07 (s, 1H), 7.37 (d, $J = 8.0$ Hz, 2H), 7.28 (d, $J = 8$ Hz, 2H), 3.74-3.71 (m, 4H), 3.48-3.45 (m, 4H), 2.35 (s, 3H). ^{13}C NMR ($CDCl_3$, 300 MHz): δ 157.90, 146.95, 138.90, 138.09, 130.88, 129.21, 124.90, 117.99, 66.62, 49.11, 21.07. LCMS: m/e 306(M+1). Anal. Calcd for $C_{15}H_{16}ClN_3O_2$: C, 58.92; H, 5.27; N, 13.74. Found: C, 58.82; H, 5.20; N, 13.80%.

5-(2,2-dimethylhydrazinyl)-4-chloro-2-p-tolylpyridazin-3(2H)-one(4j).

Off white solid, MP: 100-105°C, IR cm^{-1} (KBr): 3325, 2930, 2768, 1630, 1435, 1250. ^1H NMR (DMSO-d_6 , 400 MHz): δ 8.04 (s, 1H), 7.35 (d, $J = 4.0$ Hz, 2H), 7.26 (d, $J = 4.0$ Hz, 2H), 6.51 (bs, 1H), 2.62 (s, 3H), 2.40 - 2.19 (s, 6H). ^{13}C NMR (DMSO-d_6 , 400 MHz): δ 158.35, 140.75, 139.27, 135.58, 128.25, 124.68, 121.18, 107.56, 43.25, 21.19. LCMS: m/e 279(M+1). Anal. Calcd for $\text{C}_{13}\text{H}_{15}\text{ClN}_4\text{O}$: C, 56.02; H, 5.42; N, 20.10. Found: C, 56.29; H, 5.33; N, 20.05%.

4-chloro-5-(1,4-diazepan-1-yl)-2-p-tolylpyridazin-3(2H)-one(4k).

Light yellow solid, MP: 140-143°C, IR cm^{-1} (KBr): 2930, 2750, 1635, 1615, 1515, 1350. ^1H NMR (DMSO-d_6 , 400 MHz): δ 8.01 (s, 1H), 7.37 (d, $J = 8.0$ Hz, 2H), 7.26 (d, $J = 8.0$ Hz, 2H), 4.52 (bs, 1H), 3.76 - 3.71 (m, 4H), 2.68 (t, $J = 4.4$ Hz, 2H), 2.54 (t, $J = 5.6$ Hz, 2H), 2.35 (s, 3H), 1.91 - 1.88 (m, 2H). ^{13}C NMR (CDCl_3 , 300 MHz): δ 158.50, 147.34, 139.45, 137.60, 130.23, 127.31, 124.23, 108.75, 59.45, 58.35, 52.15, 51.30, 28.14, 21.01. LCMS: m/e 319 (M+1). Anal. Calcd for $\text{C}_{16}\text{H}_{19}\text{ClN}_4\text{O}$: C, 60.28; H, 6.01; N, 17.57. Found: C, 60.43; H, 6.21; N, 17.37%.

4-(5-chloro-6-oxo-1-p-tolyl-1,6-dihydropyridazin-4-yl)-piperazine-1-carboxylic acid *tert*-butyl ester(4l).

white solid, MP: 150-154°C. ^1H NMR (DMSO-d_6 , 400 MHz): δ 8.06 (s, 1H), 7.36 (d, $J = 7.6$ Hz, 2H), 7.28 (d, $J = 7.6$ Hz, 2H), 3.46-3.43 (m, 8H), 2.35 (s, 3H), 1.42 (s, 9H). ^{13}C NMR (CDCl_3 , 300 MHz): δ 157.40, 153.53, 147.55, 134.45, 134.00, 130.23, 129.31, 129.21, 121.43, 107.45, 49.45, 29.34, 23.56. LCMS: m/e 405(M+1). Anal. Calcd for $\text{C}_{20}\text{H}_{25}\text{ClN}_4\text{O}_3$: C, 59.33; H, 6.22; N, 13.84. Found: C, 59.43; H, 6.05; N, 13.70%.

Biological activities

Materials and methods

Human cell lines were requested from National Centre for Cell Science (NCCS: a National Cell Line Facility) Pune (MS), India. Human Angiogenesis I ELISA Strip Kit was purchased from Signosis, Inc (Santa Clara, CA, USA). DPPH (1,1-diphenyl-2-picryl hydrazyl), MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) were procured from Sigma-Aldrich Co. (St. Louis MO, USA). Other chemicals ascorbic acid, solvents, reagents used were of AR grade and were obtained from commercial sources.

Cytotoxicity assay

The cytotoxic potency of the amino-pyridazinone derivatives **4a-l** against selected cancer cell line was assessed by using the MTT assay [30-32]. In brief, cells were harvested and seeded into sterile 96-well plates in 100 μl of medium and permitted to adhere overnight. Afterward 20 μl of the individual compound (0.01 mM) added. Cells were incubated along with the compound for 48 h. After the incubation period, 10 μl of MTT was added to each well and the plates were again incubated at 37°C with humid atmosphere along with 5% CO_2 and 95% air for 4 h. The media was then smoothly aspirated, and 100 μl DMSO was added to dissolve the formazan crystals. The quantity of formazan product produced was measured spectrophotometrically at 570 nm using Thermo make Automatic Ex-Microplate Reader (M 51118170).

Anti-angiogenic activity

The evaluation of the inhibition of cytokines promoting tumor growth such as $\text{TNF}\alpha$, IGF1, VEGF, IL6, FGFb, TGF β , EGF and Leptin was carried out as per the previously described method [5]. In brief, the HEP3BPN 11 cells were treated with test compounds (4a, 4e, 4g) up to 48 h. After treatment, cell medium was

removed and cells were rinsed once with ice-cold PBS buffer (1X). Afterwards, the cells were thawed on ice and 100 μ l of 1X cell lysis buffer was added and incubated for 10 min with gentle shaking. The mixture was centrifuged at 3,000 RPM for 5 min and 90 μ l of supernatant was transferred to ELISA plate wells. Initially, 100 μ l of standard and each sample was added in each well and incubated for 1 h at room temperature. Subsequently, the contents were aspirated from each well followed by washing the well by adding 200 μ l of 1X assay wash buffer. The washing process was repeated for three times. 100 μ l of diluted biotin-labeled antibody mixture was added to every well and incubated again for 1 h at room temperature. The contents were washed as described above. To every well a 100 μ l of diluted streptavidin-HRP conjugate was added and incubated for 45 min at room temperature. Again the contents were washed like earlier. A 100 μ l of substrate was added to each well and again incubated for 25 min followed by addition of 50 μ l stop solution. The change in the color of the mixture from blue to yellow signifies the incidence of reaction. The optical density of each well was recorded by using Microplate reader at 450 nm within 30 minutes.

Antioxidant activity

Imbalance between oxidant and antioxidant compounds generates the free radicals and reactive oxygen species (ROS) which lead to the oxidative stress process. Oxidative stress causes the biomolecule oxidation in humans which results into series of disorders including cancer. Therefore there is increasing interest in the synthesis of novel compounds against the oxidative damage caused by free radicals [33]. In the present study, we have examined the antioxidant potential of amino-pyridazinone derivatives by using DPPH (2,2-diphenyl-1-picrylhydrazyl) [34], OH [35], and SOR (superoxide anion) radical scavenging action

[36] by a spectrometric assay. The results are summarized in Table 4.

DPPH radical scavenging activity

In brief, the reaction mixture comprises equal volume of 10^{-4} M ethanol solution of DPPH with individual concentrations of amino-pyridazinone derivatives (0.5-1mM). After 20 min incubation time the absorbance was read at 517 nm using Thermo make Automatic Ex-Microplate Reader (M51118170). Ascorbic acid was used as reference compound.

OH radical scavenging activity

OH radicals were generated by using the Fenton reaction system. The reaction cocktail contained 60 μ l of 1mM FeCl_3 , 90 μ l of 1, 10-phenanthroline, 2.4 ml 0.2 M phosphate buffer (pH 7.4), 150 μ l of 0.17 M H_2O_2 and 0.5-1 mM individual amino-pyridazinone derivative solution. Adding H_2O_2 initiated the reaction. After the 5 min incubation at room temperature, the absorbance of the mixture was observed spectrophotometrically at 412 nm. Ascorbic acid was used as a reference compound.

Superoxide anion radical (SOR) scavenging activity

The reaction mixture contained 1 ml of NBT (300 μ M), NADH (936 μ M) respectively and the individual concentrations of amino-pyridazinones (0.5 and 1 mM) in Tris-HCl buffer (100 mM, pH 7.4). The reaction was started by adding PMS (120 μ M) to the mixture. The reaction cocktail was incubated at 25 $^\circ$ C for 5 min and the absorbance was measured at 560 nm using Thermo make Automatic Ex-Microplate Reader (M 51118170).

The percent activity of DPPH, OH, SOR radical scavenging, cytotoxicity and antiangiogenesis activity was calculated using following

equation.

$$\text{Activity (\%)} = 1 - \frac{T}{C} \times 100$$

Where T = Absorbance of the test sample and
 C = Absorbance of the control sample.

Conclusion

A variety of novel 4-chloro-5-(aliphatic/cyclic saturated amino)-2-*p*-tolyl-(2*H*)-pyridazin-3-one derivatives 4a-l were prepared adopting simple and efficient methodologies. The structures of the compounds 4a-l were confirmed by their elemental analysis, IR, ^1H , ^{13}C NMR, and mass spectral data. All the compounds were screened for their cytotoxic, antiangiogenic and antioxidant activities. In case of cytotoxic study against three human cancer cell lines by MTT assay, the results revealed that, the compounds 4a, 4e, and 4g had excellent *in vitro* anticancer activity and can be used as lead compounds for developing new anticancer agents. These compounds were further evaluated for inhibition of selected cytokines and found that they are potent antiangiogenic agents. Furthermore, compounds 4a-l showed better OH radical scavenging activity when compared with the standard ascorbic acid. Thus, some of these compounds can emerge as a promising tool for further research work.

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