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ORIGINAL PAPER

Synthesis, antitumor evaluation, and molecular docking studies of indole–indazolyl hydrazide–hydrazone derivatives

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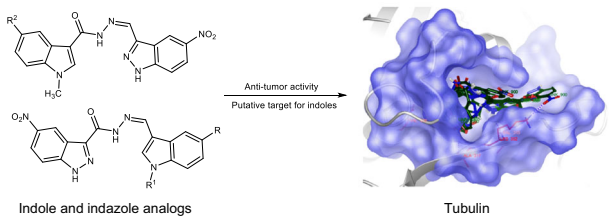
Abstract A series of ten novel hydrazide–hydrazones linked indole and indazole moieties were designed and synthesized. All the synthesized compounds were evaluated for their cytotoxicity against four human cancer cell lines (HeLa, MDA-MB-231, MCF-7, and A549). Three of the synthesized compounds showed promising cytotoxicity specifically on some of the tested cell lines with IC_{50} values ranging between 1.93 and 25.6 μM. Further, one compound was identified as a promising drug lead which showed promising cytotoxicity with IC_{50} value of 1.93 μM towards MCF-7 breast cancer cell line as compared to the standard drug doxorubicin (IC_{50} value 0.98 μM). While, all these new compounds showed no cytotoxicity on the normal human embryonic kidney cell line, HEK-293.

Graphical abstract



Keywords Indole · Indazole · Breast cancer · Tubulin · Docking

Introduction

The most ubiquitous indole ring system is widely featured in a variety of therapeutic agents with a broad spectrum of pharmacological activities which include anticancer [1–6], anti-HIV [7, 8], anti-rheumatoidal [9], and antioxidant [10]. Indole ring linked to variety of heterocyclic moieties with different functional groups has led to fascinating array of active pharmaceutical ingredients and bioactive natural products [11–14]. Most of the indole based small molecules act as potent anticancer agents, for instance indolyl glyoxalyl amide (D-24851) destabilizes microtubules and arrest the cell cycle transition specifically at G_2/M phase [15]. Similarly, the functional group such as hydrazide-hydrazone (–CO–NH–N=CH–) was also screened for different biological activities such as anti-tumor [16–19], anti-tubercular [20], anti-malarial [21], antileishmanial [22], anti-convulsant [23], antimicrobial [24], and anti-inflammatory [25]. Apart from these activities, the scientists from worldwide have reported the synthesis of different

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heterocyclic fused hydrazide–hydrazones from a medicinal chemistry perspective. Further improvements include the synthesis of oxindole hydrazides which have been reported as potent inhibitors of tubulin polymerization (IC_{50} = 0.19–0.97 μM) [26]. The hydrazide derivatives of 3-phenyl-5-sulfonamidoindole-2-carboxylic acid were reported as anti-depressant agents at a dose of 100 mg/kg [27]. 5-Chloro-3-methylindole-2-carboxylic acid benzylidenehydrazides were reported as potent apoptotic inducers by inhibiting tubulin polymerization in G_2/M phase against breast cancer cell lines T47D (IC_{50} = 0.2 μM) [28]. 3,4,5-Trimethoxybenzohydrazides were screened for their anti-tumor activity against PC3 cancer cell line [29]. Aryl hydrazones of 5-butyl-2-(4-methoxyphenyl)indole-3-carbaldehydes were strong growth inhibitor of human breast cancer cells (IC_{50}, 19–115 nM) by apoptosis and cell cycle arrest in G_2/M phase [30].

Indazole derivatives possess a broad spectrum of pharmacological activities which include anti-tumor [31–33], anti-HIV [34–36], antimicrobial [37, 38], anti-inflammatory [39–41], anti-depressant [42], anti-emetic [43], analgesic and antipyretic [44], anti-platelet [45], anti-cataract activity [46, anti-spermatogenic activity [47]. *N*-Acylhydrazones of indazole derivatives are screened for micromolar inhibitory activities against MurC and MurD enzymes from *Escherichia coli* [48]. The anticancer drugs such as ionidamine, nortopsentins, and bisindolyl hydrazide hydrazone are shown in Fig. 1.

The indole ring bearing compounds were proven to exhibit the anticancer properties, few of them were found to have slight selectivity becoming potential inhibitors of breast cancer cell lines [49, 50]. We have earlier reported the anticancer effects of the indole compounds against five cancer cell lines as shown in Fig. 2 [6, 51].

Further, the cytotoxicity studies of novel indole and indazole compounds against human cervical, breast, and lung cancer cell lines were carried out as a continuation to the previous work. The literature reports suggested that the compounds consisting of the indole nucleus may interfere with the functioning of the tubulin polymerization [52–54]. The indole bearing anticancer agents include vinblastine, vincristine, vinflunine, and hemiasterlin-A and B have been used as tubulin inhibitors. Therefore, the tubulin was selected as a putative target for the present series of compounds [55, 56]. The molecular docking has been carried out to know the basic interactions of the compounds with tubulin receptor.

Results and discussion

Chemistry

The synthesis of novel hydrazide–hydrazones linked between indole and indazole moieties are shown in Schemes 1 and 2. 5-Nitroindazole-3-aldehyde (**5**) was obtained by the Vilsmeier–Haack reaction of 5-nitroindazole (**4**), which undergo oxidation with $KMnO_4$ in acetone at room temperature for 6 h gave **6**. The compound **6** was refluxed with methanol and conc. H_2SO_4 over a period of 6 h to afford **7**, which was reacted with hydrazine hydrate in ethanol by refluxing for 8 h to afford the acid hydrazide **8** in good yield. Finally, the acid hydrazides (**8**, **11a** and **11b**) was condensed with different indole and indazole aldehydes **5**, **9a**–**9h** in glacial acetic acid at 90 °C for 6 h afforded novel hydrazide–hydrazones linked between indole and indazole moieties **10a**–**10h**, **12a** and **12b** in good yields. The detailed synthetic view of these compounds are given in Schemes 1 and 2.

Biological evaluation: in vitro cytotoxicity

Ten compounds were evaluated for their anticancer activity in the selected five human cancer cell lines viz. HeLa (cervical cancer cells), MDA-MB-231 and MCF-7 (human breast adenocarcinoma cells), A549 (human alveolar adenocarcinoma cells), and HEK-293 (normal human embryonic kidney cells) as per the reported standard protocol [57]. IC_{50} values can be defined as the drug concentration that produced 50 % inhibition of the cells and represented in μM. The IC_{50} values of the tested compounds ranged between 1.93 and 29.58 μM (Table 1). Among the 10 series compounds tested on different cancer cell lines, it was observed that three compounds (**10a**, **10b**, and **10c**) showed a comparatively promising cytotoxicity on different cell lines. Compound **10b** showed promising

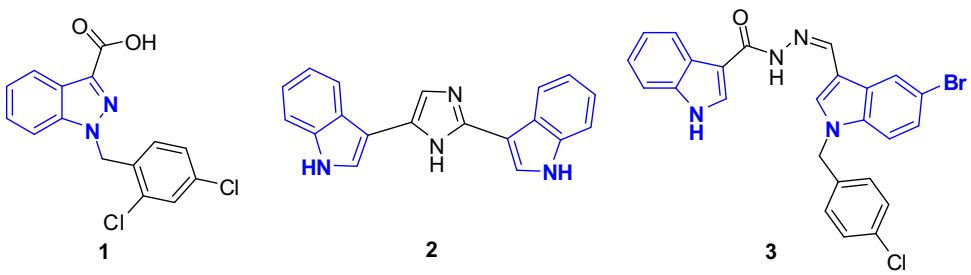


Fig. 1 Structures of ionidamine (**1**), nortopsentins (**2**) and bisindolyl hydrazide–hydrazone **3**

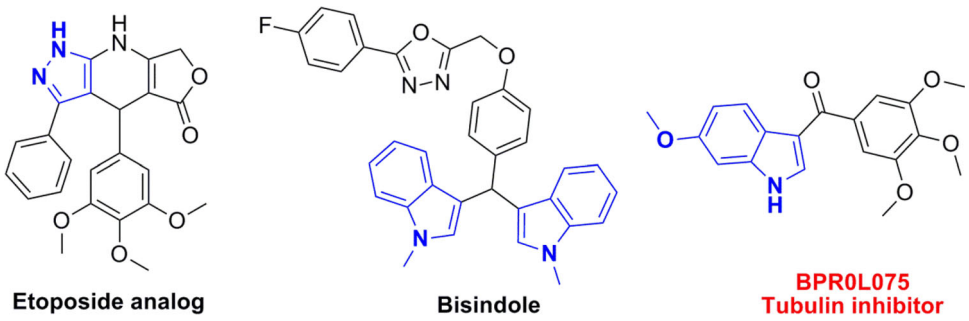
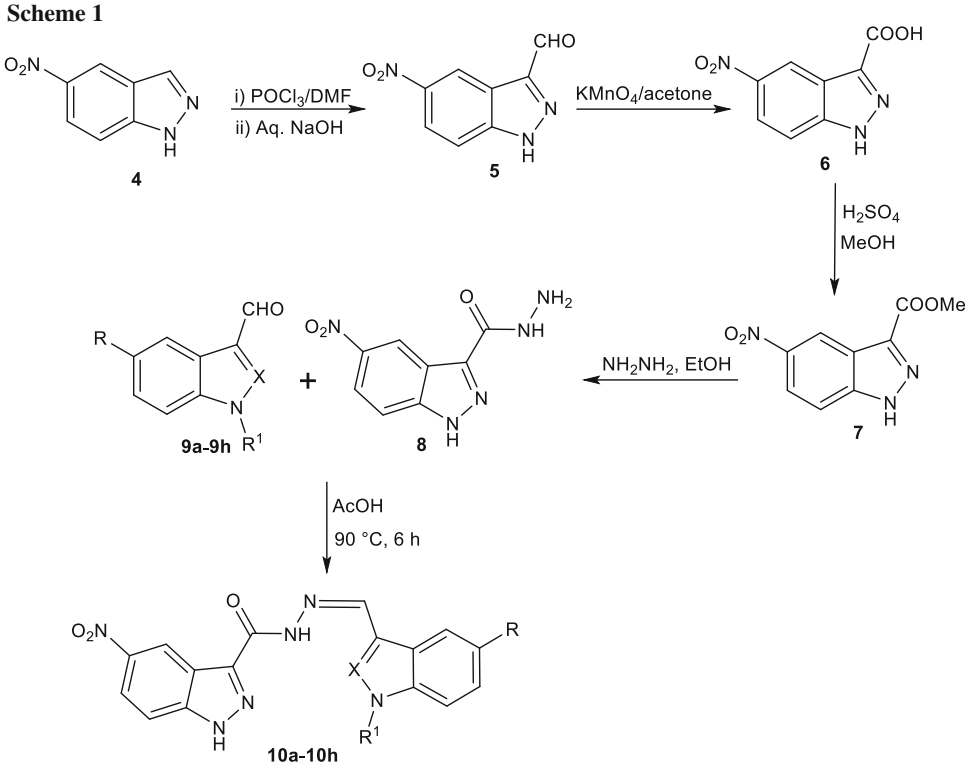


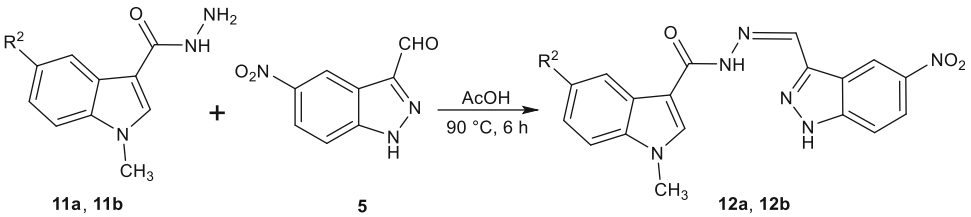
Fig. 2 Previously reported compounds and potent against breast cancer cell lines

Scheme 1



10a, R = H, R^1 = H, X = CH
10b, R = H, R^1 = CH_3, X = CH
10c, R = Br, R^1 = H, X = CH
10d, R = Br, R^1 = CH_3, X = CH
10e, R = NO_2, R^1 = H, X = CH
10f, R = OCH_3, R^1 = H, X = CH
10g, R = OCH_3, R^1 = CH_3, X = CH
10h, R = NO_2, R^1 = H, X = N

Scheme 2



12a, R^2 = H
12b, R^2 = Br

Table 1 Anti-tumor activity of the synthesized compounds

Compound	IC_{50}/μM				
	HeLa	MDA-MB-231	MCF-7	A549	HEK-293
12a	–[a]	17.08 ± 0.35	4.59 ± 0.31	–[a]	–[a]
12b	15.8 ± 0.34	–[a]	7.25 ± 0.42	29.58 ± 0.25	–[a]
10a	8.6 ± 0.35	14.25 ± 0.41	9.19 ± 0.27	5.6 ± 0.32	–[a]
10b	6.86 ± 0.28	9.88 ± 0.29	9.59 ± 0.35	7.9 ± 0.35	–[a]
10c	21.9 ± 0.35	15.09 ± 0.37	1.93 ± 0.25	25.6 ± 0.39	–[a]
10d	–[a]	–[a]	–[a]	–[a]	–[a]
10e	–[a]	–[a]	–[a]	–[a]	–[a]
10f	12.5 ± 0.32	–[a]	15.6 ± 0.54	–[a]	–[a]
10g	13.56 ± 0.35	–[a]	14.8 ± 0.31	–[a]	–[a]
10h	–[a]	11.47 ± 0.42	9.82 ± 0.37	–[a]	–[a]
Doxorubicin	0.36 ± 0.14	0.47 ± 0.4	0.98 ± 0.14	0.89 ± 0.26	

[a] Not showed any activity at maximum concentration

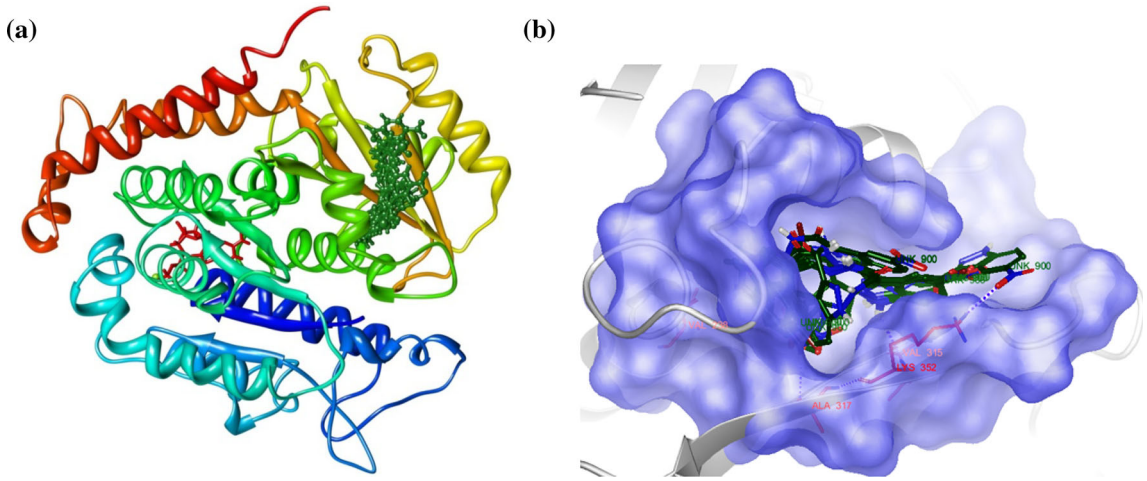


Fig. 3 **a** Tubulin receptor (PDB: 1SA0) with colchicine (*blue circle*), GDP, and Mg (*red circle*) binding cites; **b** the superimposed view of all ligands at the active site with surface (color figure online)

cytotoxic effect on HeLa and A549 cancer cell lines with IC_{50} values of 6.86 and 7.9 μM, respectively. The compound **10c** showed promising cytotoxic effect on MCF-7 cancer cell lines with IC_{50} value of 1.93 μM. While, compound **10a** showed a promising cytotoxic effect on A549 and HeLa cancer cell lines with IC_{50} values of 5.6 and 8.6 μM, respectively. It was interesting to note that all the compounds showed no activity on the normal human embryonic kidney cells, HEK-293. The compounds **10d** and **10e** also did not show any cytotoxic effect on all the tested cell lines. Further, compounds **12a** and **12b** showed promising cytotoxicity towards MCF cell line with IC_{50} values of 4.59 and 7.25 μM, respectively, while they showed moderate activity towards other tested cell lines. The compounds **10f**, **10g**, and **10h** showed moderate cytotoxicity specifically towards two cancer cell lines, HeLa and MCF-7 as compared to the doxorubicin standard drug. In case of compound **10d**, the presence of the methyl group on the bromoindole ring made it ineffective. The removal of the *N*-methyl group from bromoindole ring gave the compound **10c** which was found to be the most potent analog and was identified as a promising drug lead. The cytotoxicity data clearly indicated that the indole and indazole compounds showed selective inhibition towards the breast cancer cell lines.

Molecular docking studies

The molecular docking studies were further performed to verify the basic interactions of these compounds with the putative target, tubulin. The molecular docking studies of indole agents against tubulin receptor have been reported

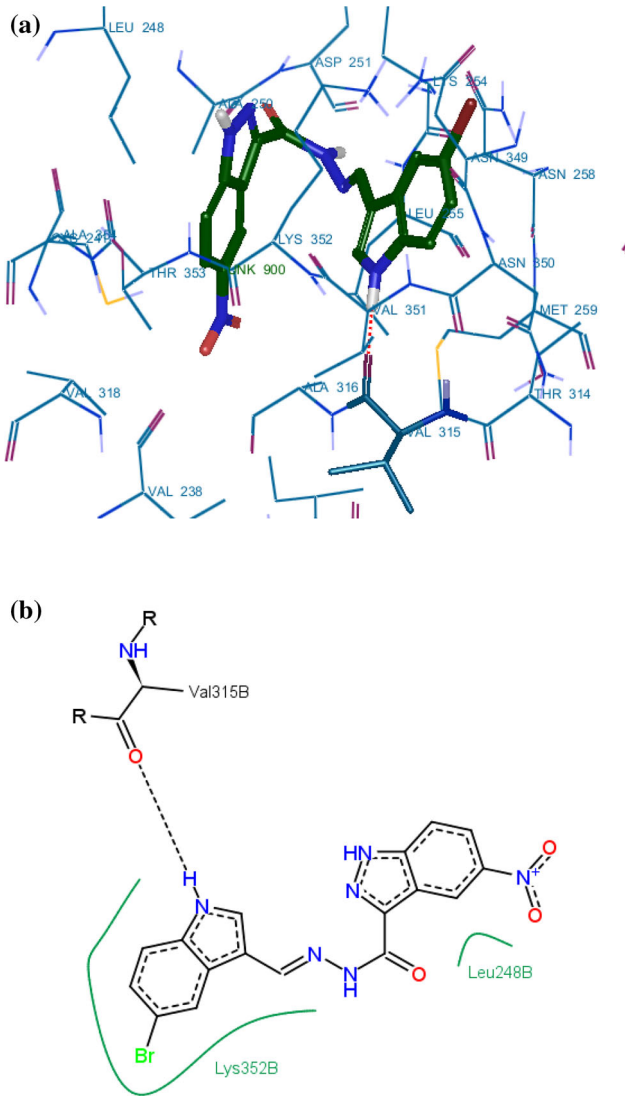


Fig. 4 **a** Compound **10c** (*green stick residue*) at active site, *red dotted line* indicates the H-bond; **b** poseview image of compound **10c** (color figure online)

several times. The current docking studies clearly present the binding modes of the indole compounds in the active site of the colchicine binding site of the tubulin receptor. The reference ligand colchicine exhibited the interactions, but the complete ligand was surrounded by the hydrophobic active site residues including Cys241,Val318, Val238, Ala316, Lys352, and Leu248 and shown in Fig. 3. The present compounds consist of both indole and indozole rings and it was observed that the binding mode of the ligands in the active site were sometimes opposite. The –NH atom of indazole ring was responsible for the backbone H-bond with residues Val315 (**12a**, **12b**, and **10c**) and with Val238 when it is bounded (**10h**) opposite to previous one. In case of compound **10d**, the presence of the methyl group on the bromoindole ring makes it ineffective. The hydrazide –NH atom was responsible for the backbone H-bond with Asn258 and a nitro group of the indazole ring exhibited the side chain H-bond with Lys352. The removal of the *N*-methyl group from bromoindole ring gave the compound **10c**; the free –NH of the indole was found to establish the backbone H-bond with Val315 and this may be the reason behind its higher potency among others in the present series. The molecular interactions of compound **10c** are presented in Fig. 4a. A similar case was observed in the compounds **10f** and **10g**, the presence *N*-methyl in compound **10g** makes it slightly less effective than compound **10f**, where *N*-methyl is missing resulted in the formation of backbone H-bond with Ala317. In case of compounds **10h** and **10e**, the compound **10h** contains two nitro substituted indazole rings on both sides and the free –NH atom is responsible for making the backbone H-bond interaction with Val238. Whereas, the compound **10e** contains nitro substituted indole and indazole ring, even though the free –NH of the indole ring exhibited the same H-bond with Val238, but it was active. The compound **10b** and **10a** did not show any interaction, but they were found to be

Table 2 In silico ADME properties of synthesized compounds

Compound	In silico ADME properties					Rule of five
	Mol wt	Po/w	logs	PMDCK	Human oral absorption	
12a	362.34	3.00	−5.58	25.80	76.98	0
12b	441.24	3.56	−6.41	67.91	80.27	0
10a	348.32	2.83	−5.21	14.53	71.86	0
10b	362.34	3.60	−5.98	27.72	81.01	0
10c	427.21	3.40	−6.06	38.50	75.18	0
10d	441.24	4.17	−6.84	73.45	84.37	0
10e	393.31	2.15	−5.42	1.46	38.41	1
10f	378.34	2.94	−5.47	14.60	72.53	0
10g	392.37	3.69	−6.20	27.87	81.59	0
10h	394.30	1.40	−5.03	0.63	28.04	1
Doxorubicin	395.25	4.62	−5.77	1114.85	100.00	0

completely surrounded by the active site residues. The present docking studies suggested that the presence of the indole ring plays a critical role in the cytotoxicity of the compounds. It also suggested that the shifting of the carbohydrazide portion from the 1*H*-indole to 1*H*-indazole does not affect the binding modes of the compounds. The Qikprop results clearly presented the in silico ADME properties and all of them followed the rule of five parameters (Table 2).

Conclusion

In continuation to our ongoing research, we designed and synthesized a series of novel hydrazide–hydrazones linked with both indole and indazole moieties. All the synthesized derivatives were evaluated for their anticancer activity against a panel of human cancer cell lines. A few compounds were observed to exhibit selective inhibition towards breast cancer cell lines. Further, the molecular docking studies were carried out against the tubulin receptor.

Experimental

All chemicals and reagents were obtained from Aldrich (Sigma-Aldrich, St. Louis, MO, USA), Lancaster (Alfa Aesar, Johnson Matthey Company, Ward Hill, MA, USA) and were used without further purification. Reactions were monitored by TLC, performed on silica gel glass plates containing 60 F-254, and visualization on TLC was achieved by UV light or iodine indicator. Proton and ^{13}C NMR spectra were recorded on a Gemini Varian-VXR-unity instrument. Chemical shifts (δ) are reported in ppm downfield from internal TMS standard. Mass ESI spectra were recorded on Micro mass, Quattro LC using ESI+ software with capillary voltage 3.98 kV and ESI mode positive ion trap detector. Melting points were determined with an electrothermal melting point apparatus.

5-Nitro-1H-indazole-3-carbaldehyde (**5**, $C_8H_5N_3O_3$)
$POCl_3$ (67.43 mmol) was added dropwise to a stirred solution of 40 cm^3 DMF at 0–5 °C over a period of 15 min. The reaction mixture was allowed on stirring for half an hour at the same temperature. 5-Nitroazaindole (10 g, 61.3 mmol) was dissolved in 10 cm^3 DMF and added dropwise to the above Vilsmeier reagent at 0–5 °C. Now the reaction mixture was allowed to stand for 3 h at room temperature. The completion of the reaction was known by monitoring the HPLC. The oily residue was poured into crushed ice and NaOH solution was added to this reaction mixture. Yellow color solid was precipitated, filtered, and recrystalized from hot ethanol. Yellow color solid; 92 % yield (10.77 g); m.p.: 215–217 °C; IR (neat): $\bar{\nu}$ = 3545, 1624, 1537, 1488, 1337, 949, 786,744 cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz): δ = 13.74 (bs, 1H, NH), 8.84 (s, 1H), 8.41 (s, 1H), 8.21–8.18 (d, 1H, J = 10.4 Hz), 7.74–7.72 (d, 1H, J = 9.2 Hz) ppm; ^{13}C NMR (DMSO-d_6, 100 MHz): δ = 110, 118, 120, 121, 136, 141, 168 ppm; MS (ESI): m/z = 192 ($[M+H]^+$).

*5-Nitro-1H-indazole-3-carboxylic acid (**6**)* [58]
$KMnO_4$ (15 g, 94.91 mmol) was soluble in 300 cm^3 water and added dropwise at room temperature to a reaction mixture of 52.35 mmol of **5** soluble in 1000 cm^3 acetone. The reaction mixture was stirred at room temperature for 6 h. Later, 50 cm^3 of 30 % H_2O_2 was added dropwise to neutralize the $KMnO_4$. The reaction mixture was filtered to remove the $KMnO_4$ and the reaction mass was concentrated by vacuum. The concentrated mass was acidified by conc. HCl, filtered, and recrystallized from methanol to afford **6**. Yellow color solid; yield 79 % (8.56 g); m.p.: 191–193 °C.

*Methyl 5-nitro-1H-indazole-3-carboxylate (**7**)* [59]
Conc. H_2SO_4 (19.323 mmol) was added drop wise to a reaction mixture of 8 g **6** (38.64 mmol) in 80 cm^3 methanol over a period of half an hour below 10 °C. The reaction mixture was refluxed for 6 h. The reaction mass was cooled to room temperature and poured into crushed ice, filtered, and recrystallized from ethanol to afford **7**. Yellow color solid; yield 64 % (5.45 g); m.p.: 234–236 °C.

5-Nitro-1H-indazole-3-carbohydrazide (**8**, $C_8H_7N_5O_3$)
Hydrazine hydrate (67.87 mmol) was added drop wise below 50 °C to a reaction mixture of 5 g **7** (22.62 mmol) in 25 cm^3 absolute ethanol. The reaction mixture was refluxed for 8 h and the reaction mass was allowed to cool to room temperature slowly. Then it was poured into crushed ice, filtered, and recrystallized from ethanol to afford **8**. Yellow color solid; yield 72 % (3.6 g); m.p.: >250 °C; IR (neat): $\bar{\nu}$ = 3479, 3327, 3211, 1726, 1673, 1608, 1535, 1468, 1349, 1242 cm^{-1}; ^1H NMR (DMSO-d_6, 200 MHz): δ = 11.86 (bs, 1H, NH), 9.88 (bs, 1H, NH), 8.63 (s, 1H), 7.93 (s, 1H), 7.21(s, 1H), 4.92 (s, 2H, NH_2) ppm; MS (ESI): m/z = 222 ($[M+H]^+$).

N′-[(E)-(1H-Indol-3-yl)methylidene]-5-nitro-1H-indazole-3-carbohydrazide (**10a**, $C_{17}H_{12}N_6O_3$)
5-Nitro-1*H*-indazole-3-carbohydrazide (**8**, 500 mg, 2.26 mmol, 1.0 eq.) was dissolved in 3 cm^3 glacial acetic acid. To this reaction mixture 2.26 mmol indole-3-aldehyde (1.0 eq.) was added and heated at 90 °C for 6 h. Later the reaction mass was neutralized with a cold $NaHCO_3$ solution, filtered, and recrystallized from ethanol to afford **10a**. Yellow color solid; yield 92 %; m.p.: 225–227 °C; IR (neat): $\bar{\nu}$ = 3372, 3200, 3060, 1652, 1618, 1540, 1432,

887,793 cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz): $\delta = 11.75$ (bs, 1H, NH), 11.57 (bs, 1H, NH), 10.92 (bs, 1H, NH), 8.95 (s, 1H), 8.38–8.32 (m, 1H), 8.22–8.13 (m, 1H), 7.91 (s, 1H), 7.78–7.76 (m, 1H), 7.51–7.41 (m, 1H), 7.24-7.08 (m, 3H) ppm; ^{13}C NMR (solid state, 125 MHz): $\delta = 177, 160, 143, 138, 137, 134, 130, 126, 125, 124, 122, 113, 112$ ppm; MS (ESI): $m/z = 349$ ($[M+H]^+$).

N′-[(E)-(1-Methyl-1H-indol-3-yl)methylidene]-5-nitro-1H-indazole-3-carbohydrazide (**10b**, $C_{18}H_{14}N_6O_3$)

This compound **10b** was prepared following the method described for the preparation of the compound **10a**, employing 500 mg **8** (1.0 eq.) with 2.26 mmol 1-methylindole-3-aldehyde (1.0 eq.) to afford **10b**. Yellow color solid; yield 90 %; m.p.: 153–155 °C; IR (neat): $\bar{\nu} = 3524, 3204, 3061, 1652, 1617, 1541, 1463, 915, 803, 730$ cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz): $\delta = 11.81$ (bs, 1H, NH), 11.23 (bs, 1H, NH), 8.37–8.11 (m, 2H), 7.88 (s, 1H), 7.73–7.71 (m, 1H), 7.53-7.46 (m, 2H), 7.31–7.13 (m, 3H), 3.84 (s, 3H) ppm; ^{13}C NMR (solid state, 125 MHz): $\delta = 174, 158, 142, 139, 138, 127, 123, 112, 35$ ppm; MS (ESI): $m/z = 363$ ($[M+H]^+$).

N′-[(E)-(5-Bromo-1H-indol-3-yl)methylidene]-5-nitro-1H-indazole-3-carbohydrazide (**10c**, $C_{17}H_{11}BrN_6O_3$)

This compound **10c** was prepared following the method described for the preparation of the compound **10a**, employing 500 mg **8** (1.0 eq.) with 2.26 mmol 5-bromoindole-3-aldehyde (1.0 eq.) to afford **10c**. Yellow color solid; yield 89 %; m.p.: 235–237 °C; IR (neat): $\bar{\nu} = 3438, 3382, 3152, 3080, 1741,1650, 1541, 1341, 892,757$ cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz): $\delta = 11.96, 11.17$ (bs, 1H, NH), 11.02 (bs, 1H, NH), 8.36–8.15 (m, 3H), 7.82 (s, 2H), 7.45-7.29 (m, 3H) ppm; ^{13}C NMR (solid state, 125 MHz): $\delta = 177, 142, 136, 130, 127, 112$ ppm; MS (ESI): $m/z = 428$ ($[M+H]^+$).

N′-[(E)-(5-Bromo-1-methyl-1H-indol-3-yl)methylidene]-5-nitro-1H-indazole-3-carbohydrazide
(**10d**, $C_{18}H_{13}BrN_6O_3$)

This compound **10d** was prepared following the method described for the preparation of the compound **10a**, employing 500 mg **8** (1.0 eq.) with 2.26 mmol 1-methyl-5-bromoindole-3-aldehyde (1.0 eq.) to afford **10d**. Yellow color solid; yield 91 %; m.p.: 206–208 °C; IR (neat): $\bar{\nu} = 3441, 3186, 3085, 1660, 1541, 1400, 1368, 885, 785$ cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz): $\delta = 11.68$ (bs, 1H, NH), 11.21 (bs, 1H, NH), 8.84 (s, 1H), 7.94–7.80 (m, 3H), 7.44 (s, 1H), 7.34–7.20 (m, 3H), 3.64 (s, 3H) ppm; ^{13}C NMR (solid state, 125 MHz): $\delta = 174, 158, 157, 139, 138, 136, 134, 128, 113, 34$ ppm; MS (ESI): $m/z = 442$ $(M+H)^+$.

5-Nitro-N′-[(E)-(5-nitro-1H-indol-3-yl)methylidene]-1H-indazole-3-carbohydrazide (**10e**, $C_{17}H_{11}N_7O_5$)

This compound **10e** was prepared following the method described for the preparation of the compound **10a**, employing 500 mg **8** (1.0 eq.) with 2.26 mmol 5-nitroindole-3-aldehyde (1.0 eq.) to afford **10e**. Yellow color solid; yield 92 %; m.p.: 300–302 °C; IR (neat): $\bar{\nu} = 3367, 3306, 3108, 3027, 1648, 1575, 1524, 1431, 827, 733$ cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz): $\delta = 11.33$ (bs, 1H, NH), 11.13 (bs, 1H, NH), 9.06 (s, 2H), 8.20 (s, 1H), 8.06–7.97 (m, 3H), 7.63 (d, 2H, $J = 8.85$ Hz) ppm; ^{13}C NMR (solid state, 125 MHz): $\delta = 177, 144, 142, 139, 137, 132, 126, 124, 121, 120, 119, 118, 116, 115, 114, 112$ ppm; MS (ESI): $m/z = 394$ ($[M+H]^+$).

N′-[(E)-(5-Methoxy-1H-indol-3-yl)methylidene]-5-nitro-1H-indazole-3-carbohydrazide (**10f**, $C_{18}H_{14}N_6O_4$)

This compound **10f** was prepared following the method described for the preparation of the compound **10a**, employing 500 mg **8** (1.0 eq.) with 2.26 mmol 5-methoxyindole-3-aldehyde (1.0 eq.) to afford **10e**. Yellow color solid; yield 84 %; m.p.: 210–212 °C; IR (neat): $\bar{\nu} = 3458, 3363, 3199, 2989, 1637, 1574, 1534, 1486, 803, 712$ cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz): $\delta = 11.54$ (bs, 1H, NH), 11.11 (bs, 1H, NH), 10.92 (bs, 1H, NH), 8.14 (s, 1H), 7.69–7.65 (m, 3H), 7.33 (d, 2H, $J = 8.5$ Hz), 6.83–6.81 (m, 2H), 3.76 (s, 3H) ppm; ^{13}C NMR (solid state, 125 MHz): $\delta = 176, 157, 141, 133, 131, 127, 117, 114, 113, 103, 55$ ppm; MS (ESI): $m/z = 379$ ($[M+H]^+$).

N′-[(E)-(5-Methoxy-1-methyl-1H-indol-3-yl)methylidene]-5-nitro-1H-indazole-3-carbohydrazide
(**10g**, $C_{19}H_{16}N_6O_4$)

This compound **10g** was prepared following the method described for the preparation of the compound **10a**, employing 500 mg **8** (1.0 eq.) with 2.26 mmol 5-methoxy-1-methylindole-3-aldehyde (1.0 eq.) to afford **10g**. Yellow color solid; yield 86 %; m.p.: 178–180 °C; IR (neat): $\bar{\nu} = 3497, 3184, 3026, 1665, 1610, 1532, 1486, 851, 795$ cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz): $\delta = 11.14$ (bs, 1H, NH), 10.96 (bs, 1H, NH), 8.11 (s, 1H), 7.68–7.62 (m, 3H), 7.39–7.34 (m, 2H), 6.91–6.87 (m, 2H), 3.77 (s, 6H) ppm; ^{13}C NMR (solid state, 125 MHz): $\delta = 174, 169, 157, 144, 142, 136, 134, 133, 126, 124, 121, 118, 115, 114, 111, 102, 57, 33$ ppm; MS (ESI): $m/z = 393$ ($[M+H]^+$).

5-Nitro-N′-[(E)-(5-nitro-1H-indazol-3-yl)methylidene]-1H-indazole-3-carbohydrazide (**10h**, $C_{16}H_{10}N_8O_5$)

The compound **10h** was prepared following the method described for the preparation of the compound **10a**, employing 500 mg compound **8** (1.0 eq.) with 2.26 mmol

5-nitroindazole-3-aldehyde (1.0 eq.) to afford **10h**. Yellow color solid; yield 89 %; m.p.: 190–192 °C; IR (neat): = 3369, 3189, 3104, 3022, 1697, 1622, 1532, 1494, 822, 748 cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz): δ = 14.23–13.17 (bs, 3NH), 8.83 (s, 1H), 8.40 (s, 2H), 8.18 (dd, 2H, J = 1.98 Hz, 9.15 Hz), 7.73 (d, 2H, J = 9.15 Hz) ppm; ^{13}C NMR (solid state, 125 MHz): δ = 189, 142, 136, 124, 121, 119, 112 ppm; MS (ESI): m/z = 395 ($[M+H]^+$).

1-Methyl-N′-[(Z)-(5-nitro-1H-indazol-3-yl)methylidene]-1H-indole-3-carbohydrazide (**12a**, $C_{18}H_{14}N_6O_3$)

The compound **12a** was prepared following the method described for the preparation of the compound **10a**, employing 500 mg compound **11a** (1.0 eq.) with 2.64 mmol 5-nitroindazole-3-aldehyde (1.0 eq.) to afford **12a**. Yellow color solid; yield 87 %; m.p.: 170–172 °C; IR (neat): $\bar{\nu}$ = 3382, 3103, 3058, 1685, 1624, 1537, 1493, 824, 747 cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz): δ = 10.93 (bs, 1H, NH), 8.69 (s, 1H), 8.12 (d, 1H, J = 6.38 Hz, 7.88 (d, 1H, J = 6.4 Hz), 7.54 (s, 1H), 7.31–7.18 (m, 5H), 3.47 (s, 3H) ppm; ^{13}C NMR (solid state, 125 MHz): δ = 192, 142, 136, 123, 121, 118, 112, 32 ppm; MS (ESI): m/z = 363 ($[M+H]^+$).

5-Bromo-1-methyl-N′-[(Z)-(5-nitro-1H-indazol-3-yl)methylidene]-1H-indole-3-carbohydrazide (**12b**, $C_{18}H_{13}BrN_6O_3$)

The compound **12b** was prepared following the method described for the preparation of the compound **10a**, employing 500 mg compound **11b** (1.0 eq.) with 1.86 mmol 5-nitroindazole-3-aldehyde (1.0 eq.) to afford **12b**. Yellow color solid; yield 85 %; m.p.: 193–195 °C; IR (neat): $\bar{\nu}$ = 3381, 3152, 3061, 1681, 1619, 1523, 1462, 790, 754 cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz): δ = 13.71 (bs, 1H, NH), 9.79 (bs, 1H, NH), 8.83 (s, 1H), 8.48–8.01 (m, 4H), 7.73–7.24 (m, 3H), 3.84 (s, 3H) ppm; ^{13}C NMR (solid state, 125 MHz): δ = 166, 141, 136, 134, 131, 128, 127, 123, 121, 118, 111, 109, 35 ppm; MS (ESI): m/z = 442 ($[M+H]^+$).

In vitro cytotoxicity assay

Cytotoxicity of the synthesized compounds was assessed on the basis of the measurement of the in vitro growth in the 96 well microtitre plates by cell-mediated reduction of tetrazolium salt to water insoluble formazan crystals by a previously described method (Mosmann 1983) [57]. Cell lines for testing in vitro cytotoxicity included HeLa derived from human cervical cancer cells (ATCC No. CCL-2), A549 derived from human alveolar adenocarcinoma epithelial cells (ATCC No. CCL-185), MDA-MB-231 derived from human breast adenocarcinoma cells (ATCC No. HTB-26), MCF7 derived from human breast adenocarcinoma cells (ATCC No. HTB-22), and HEK-293 derived from normal human embryonic kidney cells (ATCC No. CRL-1573) were obtained from American Type Culture Collection, Manassas, VA, USA. All tumour cell lines were maintained in a modified DMEM medium supplemented with 10 % fetal bovine serum, along with 1 % non-essential amino acids except L-glutamine, 0.2 % sodium hydrogen carbonate, 1 % sodium pyruvate, and 1 % antibiotic mixture (10,000 units penicillin and 10 mg of streptomycin per cm^3). The cells were washed and re-suspended in the above medium and then 100 cm^3 of this suspension was seeded into a 96-well bottom plate. The cells were kept at 37 °C in a humidified incubator (Model 2406 Shellab CO_2 incubator, Sheldon, Cornelius, OR) under a 5 % CO_2 atmosphere. After incubation for 24 h, the cells were treated for 2 days with the test compounds at concentrations ranging from 0.1 to 100 mM in DMSO and were assayed at the end of the second day. After incubation for 48 h, the cells were subjected to an MTT colorimetric assay (5 mg cm^{-3}). The effects of the different test compounds on the viability of the tumour cell lines were measured at 540 nm using a multimode reader (Infinite® M200Pro, Tecan, Switzerland). Doxorubicin was used as positive control for comparison purpose and 1 % DMSO as a vehicle control. In order to account for the toxicity of DMSO, the values obtained for the DMSO control were subtracted from those of the test compounds. Dose–response curves were plotted for the test compounds and controls after correction by subtracting the background absorbance from that of the blanks. The antitumor potency of the compounds indicated by IC_{50} values (50 % inhibitory concentration) were calculated from the plotted absorbance data for the dose–response curves. Statistical analysis was performed using GraphPad PRISM software version 3.0 (GraphPad Software, Inc, La Jolla, CA, USA). IC_{50} values (in μM) are expressed as the mean ± SD of four independent experiments. All experimental data were compared using Student's t test. In all comparisons, $p < 0.05$ was considered statistically significant.

Molecular docking studies

The molecular docking studies were performed using the Maestro 8.5 (Schrodinger LLC) installed on RHEL 5.0. The results were analyzed using the Maestro 10.1 (Academic use only). The X-ray crystallographic structure of tubulin with colchicines as reference ligand (PDB:1SA0) at resolution 3.58 Å with r value 0.223 (obs.) was downloaded from the protein data bank (http://www.rcsb.org/pdb/explore.do?structureId=1sa0) [60]. The protein was prepared by protein preparation (Schrodinger LLC).

The protein consists of the heterodimeric and homodimeric chains and among them, the chain B with colchicine was employed in the current docking studies. The chain B

consists of two different ligands, first one is colchicine and next one is guanosine-5′-diphosphate (GDP) along with metal (Mg^{+}) binding site. However, the receptor grid using Glide 5.0 was generated around the colchicine binding site. All the ligands were sketched in Chemdraw, saved in .mol files, imported in Maestro panel and prepared by Ligprep module. The extra precision docking protocol of Glide 5.0 was employed for current docking studies. The in silico ADME properties were predicted using Qikprop module from the application panel of the Meastro 8.5. The docking results were analyzed in Maestro 10.1 academic free version.

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