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Synthesis, characterization, molecular docking and cytotoxic activity of novel plumbagin hydrazones against breast cancer cells

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ABSTRACT

Novel plumbagin hydrazonates were prepared, structurally characterized and evaluated for anti-proliferative activity against estrogen receptor-positive MCF-7 and triple negative MDA-MB-231 and MDA-MB-468 breast cancer cell lines which exhibited superior inhibitory activity than parent plumbagin compound. Molecular docking studies indicated that hydroxyl groups on plumbagin and hydrazonate side chain favor additional hydrogen bonding interactions with amino acid residues in p50-subunit of NF-κB protein and these compounds inhibited NF-κB expression which may be responsible for the enhanced anti-proliferative activity. These compounds were found to be more effective against triple negative breast cancer cells and might serve as a starting point for building future strategies against triple negative breast cancers which are known for their increased drug resistance and poor prognosis of breast cancer patients.

Breast cancer is the most common type of cancer affecting more than 1 million women and causing high mortality worldwide. It is estimated that there have been 232,620 new cases of breast cancer and 39,970 deaths occurring in the USA in 2011.[1] A large number of breast cancers express estrogen receptor (ER) as well as progesterone receptor (PR)[2] and respond to hormonal therapy or aromatase inhibitors. However, there is a group of patients (12–17%) who do not respond to such treatment because of the absence of these two receptors as well as the receptor HER-2/neu (ErbB2); this group represents a highly aggressive breast cancer subtype, known as triple-negative breast cancers (TNBC),[3] that is difficult to treat. In recent years TNBC has gained attention due to its aggressive behavior, poor prognosis and lack of targeted therapies.

The current therapies for the treatment of TNBC (Fig. 1) include inhibitors of EGFR (HER1)/tyrosine kinase (Gefitinib), topoisomerase II-α (Etoposide), PARP (Poly-(ADP-ribose)-polymerase) (Olaparib), Src kinase (Dasatinib) and VEGF/VEGFR (Bevacizumab). Recently, a new approach under a clinical trial has included the use of microtubule inhibitor, viz. Ixabepilone.[4,5] Gene mapping studies have identified two major breast cancer pre-disposing genes for TNBC, BRCA1 (chromosome 7q21) and BRCA2 (chromosome 13q12),[6,7] which are found to be involved in DNA repair, cell cycle checkpoint control through regulation of p53 activity and maintenance of global chromosome stability.[8] Some studies have indicated that breast cancers in patients with BRCA1 germline mutation are more often negative for ER, PR and HER2 and likely to be positive for p53 protein, compared with controls.[9] PARP-inhibitors[10] like Olaparib, Iniparib, Veliparib and platinum-based drugs like cisplatin[11,12] are a few reported therapeutic agents for BRCA-1 or -2 associated breast cancers.

A phytochemical isolated from *Plumbago zeylanica*,[13] viz. Plumbagin (**1**) has been shown to induce apoptosis in hormone responsive as well as hormone non-responsive TNBC breast cancer cell lines in our earlier study.[14] The compound is one of the simplest plant secondary metabolites belonging to three major phylogenic families, viz. *Plumbaginaceae*, *Droseraceae* and *Ebenceae*, which also exhibits antioxidant,[15] anti-inflammatory,[16] anticancer,[14,17] antibacterial[18] and antifungal[19] activities. We have summarized the biological activities of plumbagin and its derivatives in a recent review.[13] The compound is believed to act via generation of reactive oxygen species (ROS) which subsequently damage DNA and enhance cell death.[20] Plumbagin inhibits the ER-signaling pathway by up-regulating the expression of 46 kDa isoform of ER-α, which is reported to cause inhibition of HER66-kDa mediated transactivation leading to inhibition of estrogen-mediated cell growth.[21] The hydroxyl group of plumbagin is crucial for its activity especially towards inhibition of histone acetyltransferase activity of p300/CBP, which is a

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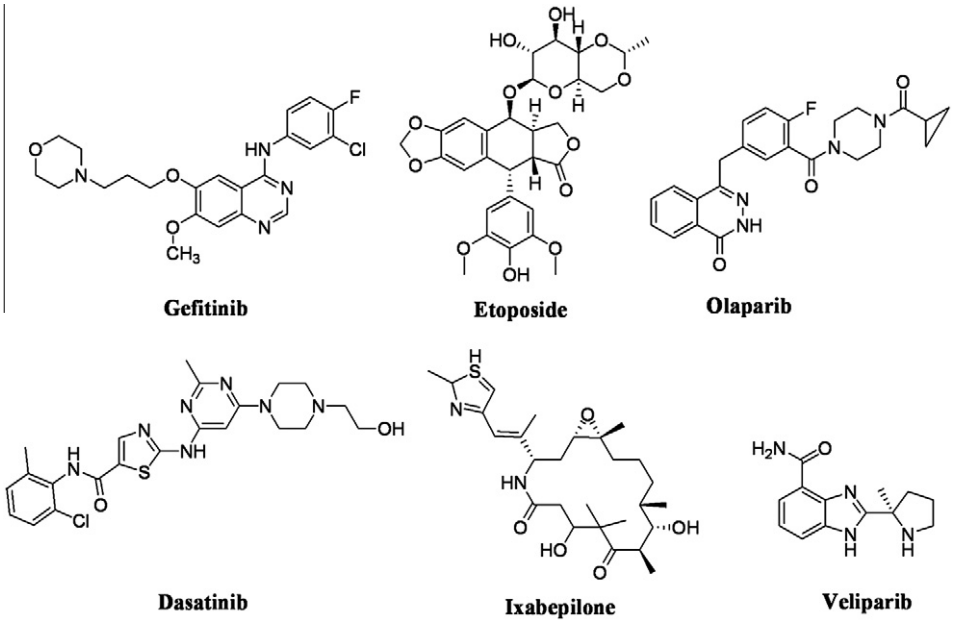
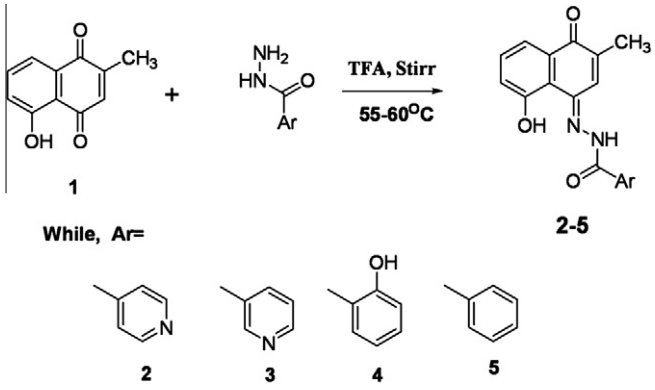


Figure 1. Clinically available compounds for treatment of triple negative breast cancer.



Scheme 1. Schematic representation of synthesis of plumbagin hydrazides (**2**–**5**).

transcriptional activator of ER-α.[22] In spite of such promising activities, the compound has remained less explored structurally for optimization of anticancer activity except for some C-3 substituted derivatives cyano, chloro, bromo, *N*-acetyl amino acids moieties[23–27] and their metal complexes.[28–31]

In the present work, we have extended our structural studies on plumbagin modification of C-4 carbonyl with hydrazide pharmacophore which has been known to induce inhibition of NF-κB activation with concomitant down-regulation of Bcl-2 expression. In our earlier study, we found that plumbagin efficiently induced apoptosis in breast cancer cells with concomitant down-regulation of Bcl-2 expression and NF-κB activity.[14] Hence, in the present work, we have selected NF-κB as a target protein for docking studies of plumbagin hydrazides in p50-subunit of NF-κB protein and have evaluated their anti-proliferative activities against hormone responsive and non-responsive breast cancer cell lines.

The compounds **2–5** were synthesized[32] as shown in Scheme 1 by condensation reactions of equimolar quantities of plumbagin (**1**) and appropriate hydrazide in absolute methanol in presence of trifluoroacetic acid as catalyst with continuous stirring at 55 °C for 4 h. The precipitated hydrazones were washed 3 times with cold methanol, dried under vacuum and purified by column chromatography using chloroform: methanol as solvent system.

All synthesized compounds were yellow to orange in color while compositional and spectral data on them is summarized in Table 1. Infra-red spectra for compounds **2–5** showed medium intensity band due to azomethine stretch in the region 1589–1604 cm^{-1}[33] confirming the formation of the hydrazonates. The broad band in the range of 3109–3269 cm^{-1}[34] is assigned to hydroxyl stretch. The compounds exhibited sharp intense bands at 1640–1647 and 1672–1683 cm^{-1} due to free quinone carbonyl and hydrazide carbonyl groups respectively. The aromatic C=C stretch was observed in the region 1519–1541 cm^{-1}, while the hydrazinic N–N stretch[33] occurred in the range 1040–1058 cm^{-1}. A phenolic C–O vibration could be observed at 1229 cm^{-1} for free plumbagin ligand which was shifted to higher frequency region 1274–1284 cm^{-1} in the hydrazonate derivatives.[31] The mass spectroscopic analysis showed molecular ion peaks corresponding to M^+ and M^++H ions at 308.10, 308.10, 322.09 and 307.12,

Table 1
Compositional and spectral data on plumbagin hydrazides (**2**–**5**)

Code	Mol. formula	Electronic spectra (nm)	IR frequencies (cm^{-1})					Mass spectra	
			O–H	C=O	C=N	C–O	N–N	Calcd	Found
1	$C_{11}H_8O_3$	394.0	3445	1663, 1645	–	1229	–	–	–
2	$C_{17}H_{13}N_3O_3$	344.5	3213–3109	1672, 1640	1604	1282	1058	307.30	308.10
3	$C_{17}H_{13}N_3O_3$	351.5	3194–3157	1680, 1647	1589	1274	1040	307.30	308.10
4	$C_{18}H_{14}N_2O_4$	373.5	3269–3234	1683, 1641	1604	1280	1055	322.32	322.09
5	$C_{18}H_{14}N_2O_3$	346.5	3195–3157	1680, 1645	1599	1284	1051	306.32	307.12

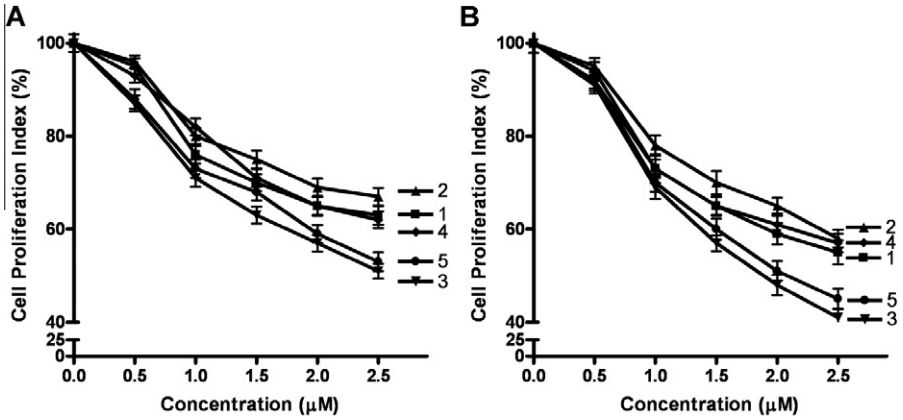


Figure 2. Anticancer activity of plumbagin hydrazides against (A) MCF-7 and (B) MDA-MB-231 breast cancer cell lines. Cells were treated for 48 h with the compounds before analysis through MTT assay.

Table 2
IC_{50} values (μM ± S.E.) of compounds **1–5** against breast cancer cell lines, as determined by MTT assays

Cell line			
Code	MCF-7	MDA-MB-231	MDA-MB-468
1	5.5 ± 0.54	3.5 ± 0.34	2.5 ± 0.33
2	6.2 ± 0.45	3.3 ± 0.46	3.3 ± 0.42
3	2.7 ± 0.32	1.9 ± 0.28	1.9 ± 0.25
4	5.3 ± 0.41	3.8 ± 0.42	2.9 ± 0.32
5	2.8 ± 0.26	2.1 ± 0.34	2.0 ± 0.31

respectively, confirming the formation of hydrazonate species proposed for compounds **2–5**. The electronic spectra showed strong intense intra-ligand absorptions in the region 344–373 nm ascribable to π–π* and n–π* transitions. A signal observed in the ^{1}H NMR spectra of the compounds at 2.10–2.12 ppm is due to the methyl group.[30] The two peaks observed at 12.31–12.52 ppm and 12.87–12.98 ppm disappeared upon D_2O exchange confirming the presence of –NH and hydroxyl groups. The hydroxyl proton of plumbagin showed an up-field shift upon formation of hydrazonates, while methylidene proton (C-3) exhibited a down-field shift appearing at 7.89–8.22 ppm. The protons of the aromatic ring of

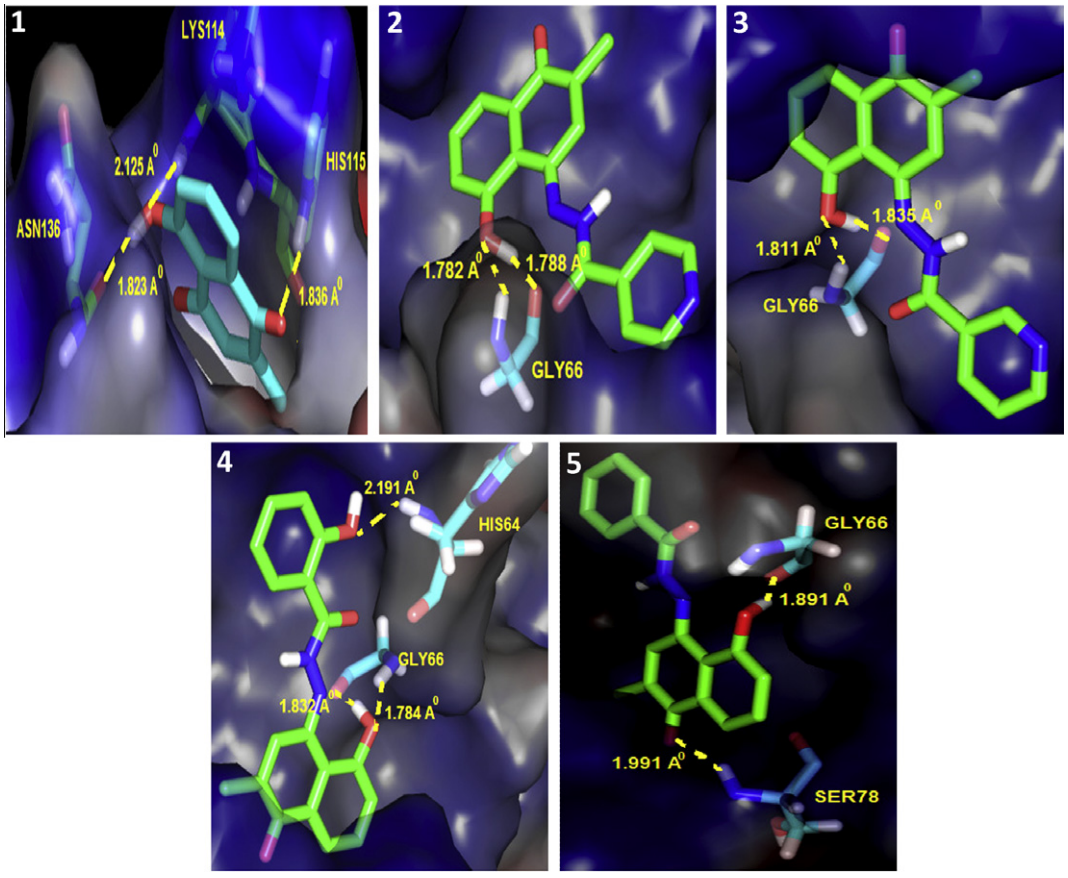


Figure 3. Binding of plumbagin hydrazides into the active site of p50 subunit of NF-B, as assessed by computer modeling studies.

Table 3
Docking results and consensus scores of synthesized plumbagin hydrazides

Code	Docking energy	Binding energy	Hydrogen bonding residues	Bond distance (Å)	No. of hydrogen bonds	Log*P*
1	−7.71	−7.68	LYS114-OA, ASN136-HA, HIS115-OA	2.125, 1.823, 1.836	3	0.89
2	−7.78	−7.77	GLY66-OA, GLY66-HA	1.788, 1.782	2	2.86
3	−7.52	−7.43	GLY66-HA, GLY66-OA	1.811, 1.835	2	3.13
4	−7.69	−7.66	GLY66-HA, GLY66-OA, HIS64-OA	1.784, 1.832, 2.191	3	4.17
5	−7.93	−7.88	GLY66-HA, SER78-OA	1.782, 1.991	2	4.00

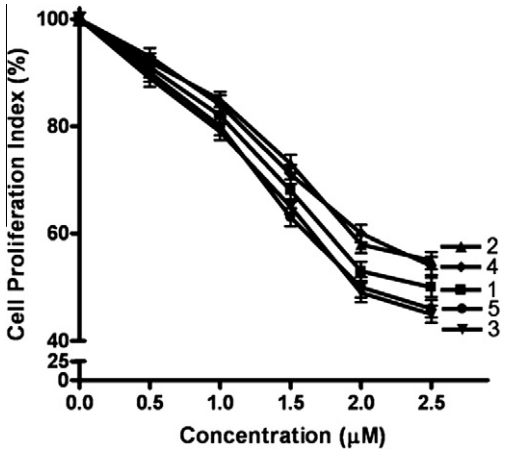


Figure 4. Anticancer activity of plumbagin hydrazides against another TNBC cell line, MDA-MB-468. Cells were treated for 48 h with the compounds before analysis through MTT assay.

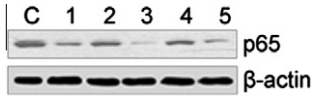


Figure 5. Representative Western blot analysis showing the effects of compounds **1**–**5** on NF-κB p65 subunit in MDA-MB-231 breast cancer cells. Cells were treated with 1.5 μM compounds for 48 h. C: vehicle-treated control cells. β-Actin protein was used as protein loading control.

plumbagin and hydrazides showed sharp peaks at 7.23–7.61 and 7.06–9.15 ppm, respectively. ^{13}C NMR spectra of all plumbagin hydrazides showed disappearance of the signal at 190.3 ppm due to C-4 carbonyl of plumbagin[30] while exhibiting a new signal at 157.3–157.8 ppm ascribed to imino carbon confirming the formation of hydrazonates at C-4 position. The C-5 hydroxyl group exhibit shifted from 161.2 to 163.2–165.2 region[31] whereas the aromatic carbon atoms appeared in the range of 115.9–152.8 ppm. The methylidenic carbon (C-3) was located between 139.4 and 141.7 ppm.[30]

The anti-proliferative activity of plumbagin and its hydrazonates was evaluated against MCF-7 (ER-positive) and MDA-MB-231 (ER-negative, TNBC) breast cancer cell lines[35,36] (Fig. 2). All compounds exhibited growth inhibitory activity against both the cell lines at significantly lower concentrations which are achievable at tissue level. The data from MTT assays was used to calculate IC_{50} values of the compounds (Table 2). NF-κB is one of the major targets of plumbagin contributing to its anticancer property.[14] The mammalian NF-κB constitutes of five polypeptide sub-units, p50, p52, p65 (RelA), RelB and c-Rel with commonly observed homodimers p50 and heterodimer p50/p65, respectively.[34] Direct binding of the inhibitor to the p50-subunit of target NF-κB is an important feature of known inhibitor molecules such as andrographolide[37] and selencotyanate[38] that form covalent adducts with Cys-62 residues of p50. Hence, we evaluated NF-κB inhibitory potential of compounds **1–5** by performing molecular docking studies on them in the p50-subunit of NF-κB protein cavity.[39] Compounds **2–5** were found to dock into the active site of p50-subunit of NF-κB with good fit confirming that hydrazide substitution does not introduce any major steric change in the parent plumbagin moiety except allowing additional hydrogen bonding interactions (Fig. 3, Table 3). The higher log*P* values observed for the hydrazonate derivatives indicate that they can easily internalize through cell membrane. Their higher binding energies were in the range of −7.43 to −7.88 kcal/mol, which are higher than those observed for the parent plumbagin compound, indicate tight binding in the active site of p50-subunit of NF-κB protein promoted through hydrogen bonding interaction with GLY66 (1.784 Å), GLY66 (1.832 Å), HIS64 (2.191 Å) residues, respectively.

Compounds **3** and **5** were especially potent against TNBC cancer cell line MDA-MB-231 (IC_{50} values 1.9 and 2.1 μM, respectively) (Fig. 2 and Table 2) which prompted us to check the activity of compounds in yet another TNBC cell line, MDA-MB-468. We found that the compounds were active against this TNBC cell line as well (Fig. 4). The degree of anti-proliferative effect of the compounds matched their effect against another TNBC cell line, MDA-MB-231 as shown above in Figure 2 and the IC_{50} values of the most effective compounds **3** and **5** was almost similar against MDA-MB-231 and MDA-MB-468 cells (Table 2). This further supports our observation that the compounds are particularly active against the hormone-insensitive breast cancer cells. Our previous work established a dose-dependent inhibition of NF-κB activity by plumbagin[14] and results presented above (Fig. 3) suggested an effective docking of compounds in NF-κB. Therefore, we assessed the direct effect of compounds on NF-κB p65-subunit in MDA-MB-231 cells. Western blot analysis clearly demonstrated an inhibition of NF-κB by the compounds (Fig. 5). While plumbagin (**1**) inhibited NF-κB as expected,[14] other derivatives were also observed to inhibit NF-κB to varying extents. In particular, compounds **3** and **5** were found to be more effective than the parent compound in inhibiting NF-κB expression. In summary, present work indicates that plumbagin hydrazonates might serve as a starting point for building future strategies against hormone non-responsive triple negative breast cancers, which are well known for their drug resistant characteristics and for poor prognosis of breast cancer patients.

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32. *Synthesis of Plumbagin Hydrazides*: The ligands (**2**–**5**) was synthesized by condensation of equimolar quantities of plumbagin and isonicotyl, nicotyl, salicyl and benzoyl hydrazide respectively in methanol in presence of trifluoro acetic acid as catalyst with continuous stirring at 60 °C for 4 h. The precipitated compounds were washed 2–3 times with cold methanol, dried under vacuum and purified by column chromatography using chloroform: methanol (9:1) as solvent system.
2 ((11E)-N′-(5-hydroxy-2-methyl-1-oxonaphthalen-4(1H)-ylidene)isonicotinohydrazide), yield: 68%, UV–vis (DMSO): λ nm:(344.5). IR (KBr disk), ν, cm^{-1}: 3213–3109 (–OH), 1672, 1640 (–C=O), 1604 (C=N), 1282 (C–O), 1058 (N–N). ^1H NMR (500 MHz, DMSO-d_6) (δ, ppm) 2.13 (s, 3H, –CH3), 7.27 (d, J = 10.0 Hz, 1H), 7.52 (t, J = 9.5 Hz, 1H), 7.61 (d, J = 9.0 Hz, 1H), 7.92 (d, J = 6.0 Hz, 2H), 8.18 (s, 1H), 8.83 (d, 7.0 Hz, 2H), 12.52 (s, 1H, D_2O exchangeable), 12.88 (s, 1H, D_2O exchangeable). ^{13}C NMR (125 MHz, DMSO-d_6) (δ, ppm) 16.43, 115.91, 117.83, 122.16, 124.84, 130.98, 131.23, 139.43, 141.25, 150.21, 157.42, 163.44, 184.13. ESIMS (m/z): Calcd 307.30, found 308.10 (M^++H), in accordance with MF $C_{17}H_{13}N_3O_3$.
3((11E)-N′-(5-hydroxy-2-methyl-1-oxonaphthalen-4(1H)-ylidene)nicotinohydrazide), yield: 64%, UV–vis (DMSO): λ nm:(351.5). IR (KBr disk), ν, cm^{-1}: 3194–3157 (–OH), 1680, 1647 (–C=O), 1589 (C=N), 1274 (C–O), 1040 (N–N). ^1H NMR (500 MHz, DMSO-d_6) (δ, ppm) 2.10 (s, 3H, –CH3), 7.23 (d, J = 8.0 Hz, 1H), 7.48 (t, J = 8.0 Hz, 1H), 7.56 (d, J = 7.0 Hz, 1H), 7.61 (dd, J = 4.5 Hz, 1H), 8.16 (s, 1H), 8.34 (d, J = 7.5 Hz, 1H), 8.82 (d, J = 4.5 Hz, 1H), 9.15 (s, 1H), 12.43 (s, 1H, D_2O exchangeable), 12.88 (s, 1H, D_2O exchangeable). ^{13}C NMR (125 MHz, DMSO-d_6) (δ, ppm) 16.43, 116.00, 117.74, 122.07, 123.53, 124.78, 128.20, 130.95, 131.06, 136.27, 141.06, 144.67, 149.24, 152.82, 157.37, 163.29, 184.11. ESIMS (m/z): Calcd 307.30, found 308.10 (M^++H), in accordance with MF $C_{17}H_{13}N_3O_3$.
4((11E)-2-hydroxy-N′-(5-hydroxy-2-methyl-1-oxonaphthalen-4(1H)-ylidene) benzohydrazide), yield: 66%, UV–vis (DMSO): λ nm: (373.5). IR (KBr disk), ν, cm^{-1}: 3269–3234 (–OH), 1683, 1641 (–C=O), 1604 (C=N), 1280 (C–O), 1055 (N–N). ^1H NMR (500 MHz, DMSO-d_6) (δ, ppm) 2.12 (s, 3H, –CH3), 6.99 (dd, J = 7.5 Hz, 1H), 7.03 (d, J = 8.0 Hz, 1H), 7.23 (d, J = 8.0 Hz, 1H), 7.47 (dd, J = 7.5 Hz, 1H), 7.51 (dd, J = 8.0 Hz, 1H), 7.60 (d, J = 7.5 Hz, 1H), 7.88 (d, J = 7.5 Hz, 1H), 7.89 (s, 1H), 11.42 (s, 1H, D_2O exchangeable), 12.31 (s, 1H, D_2O exchangeable), 12.87 (s, 1H, D_2O exchangeable). ^{13}C NMR (125 MHz, DMSO-D6) (δ, ppm) 17.08, 116.68, 117.40, 118.27, 119.94, 122.52, 124.40, 130.78, 131.39, 131.50, 134.45, 141.70, 157.61, 157.80, 164.37, 184.64. ESIMS (m/z): Calcd 322.32, found 322.09 (M^+), in accordance with MF $C_{18}H_{14}N_2O_4$.
5 ((11E)-N′-(5-hydroxy-2-methyl-1-oxonaphthalen-4(1H)-ylidene)benzohydrazide), yield: 58%, UV–vis (DMSO): λ nm:(346.5). IR (KBr disk), ν, cm^{-1}: 3195–3157 (–OH), 1680, 1645 (–C=O), 1599 (C=N), 1284 (C–O), 1051 (N–N). ^1H NMR (500 MHz, DMSO-d_6) (δ, ppm) 2.12 (s, 3H, –CH3), 7.24 (d, J = 8.0 Hz, 1H), 7.49 (t, J = 7.5 Hz, 1H), 7.58 (d, J = 7.5 Hz, 1H), 7.67 (t, J = 7.5 Hz, 3H), 8.01 (d, J = 7.0 Hz, 2H), 8.22 (s, 1H),12.33 (s, 1H, D_2O exchangeable), 12.98 (s, 1H, D_2O exchangeable). ^{13}C NMR (125 MHz, DMSO-d_6) (δ, ppm) 16.90, 116.70, 118.16, 122.51, 125.45, 128.98, 131.44, 132.71, 133.02, 141.27, 144.68, 157.86, 165.22, 184.72. ESIMS (m/z): Calcd 306.32, found 307.12 (M^++H), in accordance with MF $C_{18}H_{14}N_2O_3$.
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35. *Cell culture*: Breast cancer cell lines MCF-7 and MDA-MB-231 were maintained in DMEM medium (Invitrogen) while MDA-MB-468 cells were maintained in RPMI culture medium (Invitrogen). Both the culture media contained penicillin (50 U/mL), streptomycin (50 μg/mL) and 10% fetal calf serum. All cells were cultured in a 5% CO_2-humidified atmosphere at 37 °C.
36. *Cell Growth Inhibition Studies by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium Bromide (MTT) Assay*: Cells (3×10^3 /well) were seeded in 96-well culture plates. Each treatment had eight replicate wells and, moreover, each experiment was repeated at least three times. Test compounds were dissolved in DMSO and added to cells 24 h after seeding. At the end of treatment, MTT (0.5 mg/mL) was added and plates incubated at 37C for 2 h followed by replacement of media with DMSO at room temperature for 30 min. Ultra Multifunctional Microplate Reader (TECAN) was used to record the absorbance. 2012.
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39. *Docking studies:* All the docking calculations were carried out with AutoDock 4.0 software to analyze ligand interactions with the crystal structure binding site of p50-NFêB obtained from PDB ID (1NFK). AutoDock calculates a rapid energy evaluation through pre-calculated grids of affinity potentials with a variety of search algorithms to find appropriate binding positions. The active site of the enzyme was defined to include residues 59–71. The 3-D grid box has been generated with a grid centre co-ordinates comprising of grid spacing 0.375 Å and $60 \times 60 \times 60$ point size considering active site residues included within it. Stable docking conformation of compounds achieved by implementing energy minimization parameter AMBER force field until the gradient convergence value of 0.05 Kcal/mol was reached with distance-dependent dielectric function (€=4r). New designed all plumbagin analogs, as well as PL were placed in grid box of p50 for docking process. Customized docking parameters were set in AutoDock for best results for understanding interaction studies with new designed compounds. Parameter settings were set to 1500 iterations, 50 population sizes, 100.0 kcal/mol of energy threshold for pose generation, 300 simplex evolution steps and 1.0 neighbor distance factor. For preparing the AutoDock docking parameter file we used default settings (genetic algorithm parameters: population size = 150, number of energy evaluations = 2.5×10^7, rate of gene mutation = 0.02, rate of crossover = 0.8, maximum number of generations = 27000, number of GA runs = 10, initial dihedrals were randomly specified, elitism value was set to 1). Prior to docking, total Kollman and Gasteiger charges were added to the protein and the ligand. For each compound, the most stable docking model was selected based upon confirmation of best scored predicted by the AutoDock scoring function.