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Synthesis and X-ray study of dispiro 8-nitroquinolone analogues and their cytotoxic properties against human cervical cancer HeLa cells†

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A series of unique dispiro analogues containing an oxindole pyrrolidine 8-nitroquinolone hybrid has been obtained through a one-pot three-component 1,3-dipolar cycloaddition of azomethine ylides generated *in situ* from the condensation of isatins and benzylamine with (*E*)-3-arylidene-2,3-dihydro-8-nitro-4-quinolones. The structures of the newly synthesized compounds were characterized by using different spectroscopic techniques and by X-ray diffraction studies of their regio- and stereochemistry. All the synthesized compounds were screened for *in vitro* cytotoxic activity against the human cervical cancer cell line HeLa. The compounds have exhibited potent inhibition against human cervical cancer cells and insignificant toxicity to normal cells. The compounds **6d**, **6a**, **6h**, **6b**, and **6e** induced apoptosis of HeLa cells, through ROS influx. The expression levels of proteins involved in the mitochondrion-related pathways were detected, and Western blot analysis showed that apoptosis occurred *via* activation of caspase-3.

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1. Introduction

Since genesis, cancer has remained one of the major alarming health issues worldwide.^{1,2} According to the census, cancer is the second-most cause of human mortality after cardiovascular disease.^{3–5} Especially, cervical cancer is one of the rapidly proliferating tumors prevailing around the globe. Apart from the current standards of treatment for cervical cancer that include surgery and irradiation techniques, chemotherapy remains a reliable alternative approach for advanced metastatic cervical cancer therapy.⁶ However, due to a few confinements and disadvantages, such as difficulties in isolation, undesirable side effects, intricacy in synthesis, and multidrug protection, there is no assured treatment for some of the advanced cancer tumors. To overcome these limitations, a synthetic in-

vestigation of new chemotherapeutics with specified mechanistic action with improved suitability is very much warranted to take it to clinical studies.

In the therapeutic context, quinoline analogues have a wide occurrence among natural products and exhibit diversified biological activities depending on the structure type.⁷ Particularly, a large variety of quinolines are reported to exhibit substantial anti-cancer activity through a variety of mechanisms, such as inhibition of dehydrogenase 1A1 (ALDH1A1),⁸ cell cycle arrest in the G2 phase,⁹ inhibition of ataxia telangiectasia mutated (ATM) kinase,¹⁰ inhibition of topoisomerase,¹¹ inhibition of tubulin polymerization,¹² inhibition of heme oxygenase-1,¹³ and inhibition of tyrosine kinases¹⁴ *etc.* Among the numerous varieties of natural products, we found that the 2,3-dihydro-4-quinolone ring system exhibits interesting pharmacological properties.^{15–17} Especially, nitro groups attached to quinolines have shown considerable influence on inhibiting cancer cell growth.^{18–20} Recently, we reported such types of 8-nitroquinolone derivatives which have specific biological activities, like cell reinforcement and antibacterial and anticancer activities.²¹

Further, the pronounced anticancer activity of structurally diverse spiro pyrrolidine oxindoles was described in a number of recent publications.^{22–24} The spirooxindole-pyrrolidine core exhibits various biological functions, such as targeting tubulin polymerization,²⁵ inhibition of PLK4,²⁶ inhibition of

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the mammalian cell cycle at the G2/M phase,²⁷ inhibition of p53-MDM2,^{28,29} etc. Based on these observations our strategy was oriented towards the synthesis of a novel quinoline encapsulated spiro oxindole system.

Despite the presence of various synthetic methodologies, the multicomponent 1,3-dipolar cycloaddition of electron-deficient alkenes with azomethine ylides seems very attractive to access spiro pyrrolidine containing compounds due to the simplicity, mild reaction conditions, and atom economy.³⁰ In particular, azomethine ylides generated *in situ* from the decarboxylative condensation of α -amino acids and isatins are the most common. Herein, we report the multicomponent 1,3-dipolar cycloaddition of (*E*)-3-arylidene-2,3-dihydro-8-nitro-4-quinolones with azomethine ylides generated *in situ* from isatins and benzylamine to afford a new series of dispiro 8-nitroquinolone analogues. All the synthesized compounds were examined for their regioselectivity, high yielding MCR conversion, and cytotoxic impacts on the human cervical cancer HeLa cell line by MTT assay. The toxicity of selected potent compounds was evaluated by their cell morphology and cell death that were assessed by staining methods that clearly indicate cell death that was actuated by apoptosis. We also evaluated the potential cytotoxic mechanism of the selective potent dispiro 8-nitroquinolone analogue **6d** that involved a caspase-3 dependent apoptosis.

2. Results and discussion

2.1 Chemistry

The syntheses of dispiro 8-nitroquinolone analogues **6a-l** were achieved through a 1,3-dipolar cycloaddition reaction protocol. Initially, the dipolarophiles **3a-d** were synthesized by a Knoevenagel condensation of 8-nitro-2,3-dihydro-1*H*-quinolin-4-one **1** with aromatic aldehydes **2a-d** in the presence of a pyrrolidine base in ethanol affording (*E*)-3-arylidene-2,3-dihydro-8-nitro-4-quinolones **3a-d** in quantitative yields.³¹ In the next step, a one pot three-component 1,3-dipolar cycloaddition reaction was carried out between (*E*)-3-arylidene-2,3-dihydro-8-nitro-4-quinolones **3a-d**, and the dipole generated from isatin derivatives **5a-c** and benzylamine **4** (Scheme 1).

In order to optimize the reaction conditions, a one-pot three-component reaction of (*E*)-3-(4-bromobenzylidene)-8-

nitro-2,3-dihydroquinolin-4(1*H*)-one **3b** (1 mmol), benzylamine **4** (1.2 mmol) and 5-chloroisatin **5a** was chosen as a model reaction, kept under reflux conditions in various solvents (Table 1). From the optimization study, we observed that the best result was obtained when the methanol solvent was used under reflux conditions for 2 h, which provides dispiro oxindole pyrrolidine 8-nitroquinolone **6b** with an excellent yield (96%) (Table 1, entry 5). We extended this further, by replacing the substituent in the aryl group (OCH₃, Cl, Br, F) and isatin (Cl, Br, F) but found no impact on the yield of the products (Scheme 1, Table 1).

With the optimized results in hand, all the subsequent cycloaddition reactions were explored by refluxing a mixture of (*E*)-3-arylidene-2,3-dihydro-8-nitro-4-quinolones **3a-d** (1 mmol), benzylamine **4** (1.2 mmol) and isatin **5a-c** in MeOH for 2–3 h.

The structure of the synthesized cycloadduct **6a-l** was characterized by IR, ¹H NMR, ¹³C NMR, HR mass spectrometry, X-ray diffraction, and elemental analysis. The IR spectrum of compound **6b** showed two intense absorption bands at 1700 and 1614 cm⁻¹, characteristic for 8-nitro-4-quinolone and the oxindole ring carbonyl functionalities, respectively. The ¹H NMR spectrum of product **6b** showed a sharp singlet at δ 10.52 and a doublet, resonating at δ 8.37, which were accounted for by isatin and quinoline NH. A multiplet at δ 4.06–3.99 accounted for quinoline N-CH₂ and the pyrrolidinylmethine proton that appeared as a doublet at δ 4.57 (J = 10.0 Hz), demonstrating the regiochemistry of the cycloaddition reaction. In the case of the other possible regioisomer **6'l**, the pyrrolidinylmethine proton would have appeared as a singlet in the ¹H NMR spectrum. In the ¹³C NMR spectrum, the peaks at δ 45.90 assignable to quinolone N-CH₂ carbon, at δ 54.07, 58.42 for pyrrolo C-H carbons, at δ 63.05, 69.78 for spirocarbons, and at 179.56, and 191.04 for amide and 4-quinolone carbonyl carbons, respectively (Fig. 1). In the HR mass spectrum, the presence of a molecular ion peak at m/z = 631.0583 confirmed the formation of **6b**. The regio- and stereochemistry of the cycloaddition product **6l** were further confirmed by single crystal X-ray analysis (Fig. 2).

A plausible mechanism for the regio- and stereoselective formation of the hybrid heterocycles **6a-l** is portrayed in Scheme 2. The azomethine ylide is **8** generated *in situ* from the reaction of isatin **4** with benzylamine **5**. The subsequent

Scheme 1 Synthesis of dispiro 8-nitroquinolone analogues **6a-l**.

Table 1 Optimization of the reaction conditions for the synthesis of compound **6b**

S. No	Solvent	Temperature	Time (h)	Yield ^a (%)
1	Toluene	Reflux	10	65
2	1,4-Dioxane	Reflux	12	63
3	Acetonitrile	Reflux	12	54
4	Ethanol	Reflux	4	83
5	Methanol	Reflux	2	96
6	Tetrahydrofuran	Reflux	10	—

^a Isolated yield after recrystallization. **Fig. 1** ¹H and ¹³C NMR chemical shift values for product **6b**.

reaction of **8** with the electron deficient β -carbon of the dipolarophile **3a-d** furnished the cycloadducts **6a-l** as a single regioisomer. Despite the presence of four stereocenters in the cycloadducts **6a-l**, this leads to the selective formation of only one of the possible diastereomers, since the electron-rich carbon of the dipole adds to the electron-deficient β -carbon of the α,β -unsaturated system *via* path A, which is not possible in path B.

X-ray crystallography. The structure of compound **6l** was further confirmed by single crystal X-ray analysis (Fig. 2).

Molecular geometry and crystal packing. The triclinic centrosymmetric lattice of compound **6l** contains two molecules in the unit cell. The convergence of the structure solution is confirmed from the R factor of 4.8%. According to a Cremer & Pople conformational analysis,³² the central five-membered ring is observed to be in an envelope conformation on a

Scheme 2 A plausible mechanism for the formation of compound **6a-l**.

nitrogen atom (N3) with the puckering values of $q_2 = 0.363(2)$ Å and $\phi_2 = 176.0(3)^\circ$. The phenyl ring is oriented with the dihedral angles of $30.2(1)$, $62.0(1)$ and $32.6(1)^\circ$ with the fluoro-phenyl, methoxyphenyl and nitrophenyl groups, respectively.

The hydrogen bonding dimensions are listed in Table 2 and the packing arrangement of the molecules is depicted in Fig. 3. Intermolecular interactions, especially classical and non-classical hydrogen bonds, play a crucial role in the formation of crystalline solids and their physiochemical properties. These hydrogen bonding interactions can be classified and notated with graph-set nomenclature which is useful in comparing the stability molecular conformations and crystalline lattices between similar molecules.³³ The molecular structure features intramolecular classical N-H $\cdots$ O and non-classical C-H $\cdots$ O interactions. The classical intramolecular hydrogen bond leads to self-associated S(6) motif.

Fig. 2 ORTEP diagram of compound **6l**.**Table 2** Hydrogen bonding geometry in **6l** crystal

D-H $\cdots$ A	<i>d</i> (D-H)	<i>d</i> (H $\cdots$ A)	<i>d</i> (D $\cdots$ A)	$\angle$ (DHA)
N(2)-H(2) $\cdots$ O(2) ^{#1}	0.86	2.32	2.982(2)	134
N(4)-H(4) $\cdots$ O(1) ^{#2}	0.86	2.03	2.872(2)	168
C(16)-H(16) $\cdots$ O(4)	0.98	2.30	2.828(2)	113
C(9)-H(9) $\cdots$ O(1)	0.98	2.58	3.147(2)	117
C(32)-H(32A) $\cdots$ O(1)	0.97	2.50	3.132(2)	123
N(2)-H(2) $\cdots$ O(2)	0.86	2.03	2.618(2)	125

Symmetry transformations used to generate equivalent atoms: #1 $-x + 2, -y + 1, -z + 1$ #2 $-x + 1, -y + 1, -z + 1$.

Fig. 3 Packing of the molecules 6l in a triclinic unit cell is viewed down along the *a*-axis. H-Bonds are shown as dashed lines.

Furthermore, the crystal packing is stabilized through C–H...O and π – π interactions. The substituted fluorine is expected to participate in the intermolecular interactions. But, it is observed to be a hydrophobic region of the crystalline lattice. The different hydrogen bond motifs with the necessary graph-set notations are depicted in Fig. 4. Through the two intermolecular N–H...O hydrogen bonds, ring $R_2^2(8)$ and $R_2^1(4)$ motifs are formed. A bifurcated or three centered N–H...O hydrogen bond is observed around the N2 donor atom.

Quantum chemical analysis. The relative stereochemistry of cycloadduct 6l was studied by the quantum chemical density functional theory (DFT) method. The frontier molecular orbital (FMO) and Fukui calculations were carried out to un-

Fig. 5 HOMO and LUMO energies of dipole 8 and dipolarophile 3c calculated at the B3LYP/6-31G* level of theory.

derstand the regioselectivity of cycloadduct 6l. The FMO calculation revealed that the energy gap between HOMO 8–LUMO 3c (6.029 eV) is higher than that of LUMO 8–HOMO 3c (4.373 eV) (Fig. 5). This suggests that the HOMO of dipole 8 (azomethine ylide) and LUMO of dipolarophile 3c interaction (normal electron demand condition) is more feasible than that of the HOMO of dipolarophile 3c and LUMO of a dipole 8 (inverse electron demand condition) due to the lower energy gap, which is in good agreement with the experimental observation.

The DFT based local chemical reactivity Fukui function parameters, for nucleophilic (f_k^+) and electrophilic attack (f_k^-), were calculated through the NBO atomic charges. From the calculated local chemical reactivity parameters (Table 3),

Fig. 4 a) Self-associated ring S(6), ring $R_2^2(8)$ b) $R_2^1(4)$ motifs: H-Bonds are shown as dashed lines.

Table 3 Local chemical reactivity Fukui function parameters

Reactant	Site	f_k^+ (a.u.)	f_k^- (a.u.)
Dipolarophile 3c	C27	0.0092	0.0028
Dipolarophile 3c	C28	0.0067	-0.1273
Azomethineylide 8	C12	-0.1817	0.0101
Azomethineylide 8	C63	-0.1656	-0.0058

the reaction mechanism was at the C63 site of azomethineylide **8** which shows an f_k^- value -0.0058 a.u. and attacks the C28 position of dipolarophile **3c** (f_k^+ value 0.0067 a.u.). Further, at the C27 site of dipolarophile **3c** the f_k^+ value was 0.0092 a.u., and it underwent nucleophilic attack on the C12 site (0.0101 a.u.) of azomethineylide **8**. These results were well matched with the experimental observation (Fig. 6).

The stability of the product **6l** was found to be more stable compared to the reactant **3c** and the corresponding total energies are found to be -1928.5826 and -1928.6386 by Hartrees at the level of theory above. Moreover, a low activation energy and reaction energy indicate that the formation of compound **6l** would be quite favored at the MO6-2X/6-31G/levels. The results indicate that the formation of compound **6l** was found to be exothermic in nature. All the computa-

tional calculations were performed using the GAUSSIAN 09W program.³⁴

The selected optimized geometrical values of theoretical (DFT) and single crystal XRD values of the product **6l** are presented in ESI† Table S2 and the corresponding correlation coefficient graphs are shown in Fig. 7. In addition to that, the selected experimentally observed geometrical values of bond length, bond angle and torsional angle values correlate well with the theoretically predicted values and the corresponding correlation coefficients were found to be 0.997, 0.925 and 0.999 at the M062x/6-31g** level of theory.

2.2 Biology

2.2.1. In vitro cytotoxicity of synthesized compounds 6a-l against the human cancer cell line. Recent reports revealed that the nitroquinolines have a significant ability to inhibit cancer cell growth. In particular, nitroquinolines were found to be most active against the cervical cancer cell line.^{19,20} Further, the oxindole and pyrrole containing heterocycles have shown promising anticancer activities against the human cervical cancer cell line HeLa.^{35,36} These reports suggest that the quinoline, pyrrole, and oxindole containing compounds

Fig. 6 The transition state structures of reactant **8** and **3c** and product **6l** optimized by the B3LYP/6-31G* level of theory.

Fig. 7 Correlation coefficient graphs of a) dihedral angle, b) bond length, and c) bond angle values with XRD values.

might possibly inhibit the growth of cervical cancer cells. Hence, we used the specified cervical cancer cell line HeLa to evaluate the *in vitro* cytotoxic effects of the newly synthesized dispiro pyrrolo oxindole 8-nitroquinolones analogues **6a–l**.

The well-known anticancer drug doxorubicin (Dox) was selected as the reference drug to compare the *in vitro* anticancer potency of the synthesized compounds by MTT [3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide] assay. The IC_{50} values of the synthesized compounds and Dox against the tested cervical cancer cell line are summarized in Table 4. The MTT experimental results have demonstrated well that all the compounds show the ability to inhibit cell proliferation in a dose-dependent concentration manner. Generally, the synthesized compounds had a broad-spectrum of anticancer activity, showing favorable inhibition against the tested cancer cell line HeLa ($IC_{50} < 27 \pm 1.5 \mu M$). Specifically, **6d** exhibited an excellent inhibition against the human cancer cell line HeLa with the IC_{50} values of $16 \pm 0.2 \mu M$ and the compounds **6a**, **6h**, **6b**, and **6e** also exhibited better activity, and the rest showed a moderate activity.

2.2.2. Structure–activity relationship (SAR). Based on the result obtained from the MTT assay, we have tried to understand the role of different substitutions on cytotoxic activity:

All the synthesized compounds **6a–l** exhibited good cytotoxic activity. This might be due to the presence of a nitro

and an oxo group on the quinoline ring and the substitution attached to the pyrrolidine group of the synthesized spiro heterocycle. As is clear from Table 4, compounds **6a**, **6b**, **6d**, **6e**, and **6h** are the most active amongst the reported compounds. Compound **6d** has an IC_{50} of $16 \pm 0.2 \mu M$ against the cervical cancer cell HeLa, which is close to that of the reference compound doxorubicin ($14 \pm 0.3 \mu M$) and similarly compound **6h** exhibited an IC_{50} value $19 \pm 0.4 \mu M$ against the HeLa cell line. From these results, we conclude that compounds substituted with an electron donating methoxy group on an aryl ring showed good activity, which revealed that the substitution on the aromatic ring plays an important role in the activity. From the comparison of compound **6d** and **6h**, it is clear that chloro substitution on an oxindole ring is more active than bromo substitution as shown in Table 4. In the case of the 4-chlorophenyl group-bearing compounds **6a** and **6e** the IC_{50} values show that compound **6a** is the more active, which also reveals that chloro substitution on an oxindole ring induces more activity than bromo substitution. From the results, the other fluoro substituted compounds show moderate activity. From the SAR studies the order of cytotoxicity effects of the compounds is **6d** > **6a** > **6h** > **6b** > **6e**.

2.2.3. Detection of cell death by morphological analysis. The potential cytotoxic effects of the compounds on HeLa cells were investigated by morphological changes and were observed under phase contrast microscopy. HeLa cells were treated with compounds **6d**, **6a**, **6h**, **6b**, and **6e** at the IC_{50} concentration for 24 h. Fig. 8 shows that after treatment of the HeLa cells, they were appeared to be swollen and detached from the neighbor cells with a completely disintegrated membrane, rounded and shrunken, which indicates that the synthesized compounds probably induce cell death at IC_{50} concentrations, whereas, untreated HeLa cells showed a healthy and normal morphology.

2.2.4. Hemolytic activity. To evaluate the hemocompatibility and non-toxicity of the compounds on normal cells, a hemolytic activity test was performed. Human blood samples (RBCs) were used for the experiment and the human blood samples were provided by Mouthe hospital, Coimbatore, Tamil Nadu. PBS and H_2O were used as the positive and

Table 4 *In vitro* cytotoxic activity by MTT assay

S. No	R	R ₁	Compound	IC_{50} of HeLa (μM)
1	Cl	Cl	6a	18 ± 1.1
2	Br	Cl	6b	21 ± 0.1
3	F	Cl	6c	27 ± 1.5
4	OCH ₃	Cl	6d	16 ± 0.2
5	Cl	Br	6e	23 ± 0.6
6	Br	Br	6f	25 ± 1.3
7	F	Br	6g	26 ± 0.4
8	OCH ₃	Br	6h	19 ± 0.4
9	Cl	F	6i	25 ± 0.5
10	Br	F	6j	26 ± 1.6
11	F	F	6k	20 ± 0.8
12	OCH ₃	F	6l	24 ± 1.6
Control			DOX	14 ± 0.3

Fig. 8 Morphological analysis of HeLa cancer cells compounds treated with **6d**, **6a**, **6h**, **6b**, and **6e** compared with untreated HeLa as the control. The morphological changes, like the shrinkage and rounding of apoptotic cells, were observed under a phase-contrast microscope.

negative controls to compare the activity. It was observed that the control solution wherein the erythrocyte suspension treated with distilled H_2O in a 1:1 ratio appeared dark red due to the release of hemoglobin (Hb) from RBC lysis. However, the erythrocyte suspension treated with compounds at IC_{50} concentrations did not appear in red which, indicates no noticeable hemolytic effect on RBC. Our present hemolytic findings validate the active compounds that exhibited a low hemolytic activity of less than 7% when used at IC_{50} concentration, whereas water as a negative control showed 100% hemolysis. The experimental results suggest that the active compounds **6d**, **6a**, **6h**, **6b**, and **6e** exhibit minimum or low toxicity to human blood cells.

2.2.5. The compound induced intracellular ROS production. The favorable potency of compounds **6d**, **6a**, **6h**, **6b**, and **6e** toward HeLa cancer cells prompted us to investigate the

preferred mechanism of action. Very recently, quinolines have been reported as anti-cancer agents by inducing oxidative stress-mediated DNA damage.³⁷ Banerjee and co-workers reported a novel bis-arylidene oxindole which generated an excess of ROS and eventually killed cancer cells *via* apoptosis.³⁸ The oxindole alkaloids were extracted from *Uncaria tomentosa*, which induced apoptosis of HT29 cells through ROS-mediated activation and DNA repair.³⁹ The above studies suggest that both quinoline and oxindole containing compounds could serve as ROS-generating agents for cancer therapy. From the literature, we assume that our synthesized molecules incorporating an oxindole and quinoline nucleus might possibly induce cell death through ROS-mediated mechanisms.

Hence, we used the DCFH-DA (2',7'-dichlorodihydrofluorescein diacetate) assay to determine the intracellular

Fig. 9 a) Detection by a fluorescence microscope of the intracellular ROS of HeLa cancer cells treated with compounds **6d**, **6a**, **6h**, **6b**, and **6e** compared with untreated HeLa as the control. b) ROS fluorescence intensity of HeLa cells after incubation of the compounds compared with a positive control H_2O_2 .

Fig. 10 Detection of apoptosis by fluorescence microscopy in HeLa cells after treatment with compounds **6d**, **6a**, **6h**, **6b**, and **6e**. The cells were stained with DAPI.

ROS levels. Treatment of HeLa cells with the selected active compounds **6d**, **6a**, **6h**, **6b**, and **6e** at IC_{50} concentrations was followed by DCFH-DA treatment for 30 min. In the presence of ROS, DCFH-DA is rapidly oxidized into fluorescence dichlorofluorescein (DCF), which increases the green fluorescence. Interestingly, as shown in Fig. 9a, the synthesized compounds enhanced the green fluorescence intensity, with the percentage of cells with green fluorescence being significantly higher than that of the control. The increase of ROS generation in the treated HeLa cells was quantified by determining the percentage of cells with a significantly increased high level of green fluorescence in a flow cytometer (Fig. 9b).

This result supports the hypothesis that the selective active compounds **6d**, **6a**, **6h**, **6b**, and **6e** induced cell death mainly through ROS-mediated mechanisms.

2.2.6. Detection of apoptotic cells by DAPI staining for nuclear damage. DAPI (4',6-diamidino-2-phenylindole) is a prevalent nuclear fluorescent stain which strongly binds to the adenine-thymine clusters of the minor section of two-fold stranded DNA and distinguishes chromatin condensation or nuclear damage. The binding of DAPI to DNA produces an enhanced fluorescence clearly owing to the removal of water molecules from both DAPI and the minor groove. DAPI stains the apoptotic cells as bright colors due to the compressed nucleus that indicates a typical apoptotic feature.

A fluorescence microscopy analysis of DAPI stained cells was undertaken to study nuclear changes and apoptotic body development, both of which are also involved in the apoptosis.⁴⁰ The cervical cancer HeLa cells treated with compounds **6d**, **6a**, **6h**, **6b**, and **6e** exhibited an apoptotic morphology. Morphological changes characterized by cytoplasmic and nuclear shrinkage, chromatin condensation and apoptotic body formation were observed after treatment, whereas the untreated control cells appeared as rounded and homogeneously stained with DAPI. The bright patches indicate condensed chromatin and apoptotic bodies that are hallmarks of early and late apoptosis (Fig. 10). In contrast, the active **6d**, **6a**, **6h**, **6b**, and **6e** compounds induced significant cell death as indicated by the DAPI staining.

2.2.7. Detection of apoptotic cells by Annexin V-FITC and propidium iodide (PI) staining and activation of caspase-3. The apoptotic effect of active compound **6d** on HeLa cells was further evaluated by flow cytometry using the dual fluorescence of an Annexin V FITC/PI (AV/PI) dual staining assay. The cells in early apoptosis bind only to Annexin V, whereas dead and late stage apoptotic cells bind to both Annexin V-FITC and propidium iodide.⁴¹ In this study, HeLa cells were treated with the compound **6d** for 48 h at IC_{50} concentrations to examine the apoptotic effect. It was observed that

Fig. 11 a) Detection of apoptosis by using the Annexin V-FITC and PI method by treating HeLa cells for 48 h with compound **6d**. Quadrants: upper left (necrotic cells); lower left (live cells); lower right (early apoptotic cells); and upper right (late apoptotic cells). b) Effect of compound **6d** induced caspase-3 activation in HeLa cells determined by Western blot analysis.

Fig. 12 a) Three-dimensional representation of the molecular interaction of compound **6d** with the protein 5X5O. b) Two-dimensional representation of the molecular interaction of compound **6d** with the protein 5X5O. c) Molecular interaction of compound **6d** with caspase-3.

the compound **6d** showed significant apoptosis against HeLa cells. The results indicated that control group of untreated cells showed no apoptotic or necrotic activities with 100% gated cells, whereas the percentage of apoptotic cells was higher in the case of cells treated with the effective compound **6d** which showed 95.68% apoptotic cells (Fig. 11a).

To investigate whether the compound **6d** induced apoptosis of the HeLa cells was mediated *via* the caspase-3 pathway, the caspase-3 content was evaluated by a Western blot analysis. Caspases are enzymes of cysteine-aspartic acid proteases and perform the well-known role of the activation of an execution of apoptosis markers.⁴² Importantly, the activation of the caspase-3 enzyme is to induce cellular DNA fragmentation which leads to apoptotic cell death.⁴³ After treatment with the selected compounds for 24 h, the western blot results (Fig. 11b) indicate the up-regulation of the apoptotic activator enzyme caspase-3, as compared to the untreated control, in HeLa cells. From these results, the apoptotic pathways activated caspase-3 leading to apoptosis in the HeLa cells.

3. *In silico* molecular docking

In order to theoretically calculate the binding mode, molecular docking studies have been carried out for the compound **6d** against the cervical cancer suppressor gene 4 protein (PDB ID: 5X5O)⁴⁴ of the HeLa cervical cancer cell line using the Glide module of Schrodinger. The active compound **6d** bounds very well with the cervical cancer protein 5X5O with a Glide score of -4.2 , kcal mol^{-1} , which shows two crucial hydrogen bonding interactions with ASP92 through the NH substituent of the oxindole ring, SER89 with C=O of the indole ring (Fig. 12b). From these results, we observed that the synthesized compound **6d** has a good mode of binding activity with the cervical cancer cell HeLa.

Based on the results of western blot analysis, the interaction of compound **6d** with caspase-3 was theoretically calculated. Compound **6d** bounds very well with caspase-3 by hydrophobic interaction (Fig. 12c). The binding energy was observed to be -6.02 kcal mol^{-1} .

4. Conclusions

In summary, we have synthesized structurally novel dispiro 8-nitroquinolone analogues *via* one pot three component 1,3-dipolar cycloaddition reactions from the corresponding (*E*)-3-arylidene-8-nitro-2,3-dihydro-4-quinolone as a dipolarophile and azomethine ylide generated from isatin and benzylamine. The standardized procedure adapted from the 1,3-dipolar cycloaddition of compounds fetched an excellent yield in a short reaction time with high regio- and stereo-selectivity. The structures of the synthesized compounds were well characterized by spectroscopic and single crystal XRD analysis. All the synthesized compounds were screened for their *in vitro* cytotoxic activity against the cervical cancer cell line HeLa. Compared to Dox, the synthesized novel compounds exhibited strong inhibition against the tested cancer cell line HeLa and were less toxic to human normal cells (human RBC cells), indicating their good selectivity toward cancer cells. The selected potent compound **6d** induced typical morphological changes and remarkable apoptosis of HeLa cells. Additionally, the results suggested that treatment of HeLa cells with the **6d** analogue increased the intracellular ROS levels and apoptosis induced by the activation of caspase-3 apoptosis. Based upon the *in silico* molecular docking results, it is clearly evident that the active compounds have good binding activity with the HeLa cell line receptor.

Conflicts of interest

There are no conflicts to declare.

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