Unveiling novel diphenyl-1H-pyrazole based acrylates tethered to 1,2,3-triazole as promising apoptosis inducing cytotoxic and anti-inflammatory agents

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ABSTRACT

Meagre and suboptimal therapeutic response along with the side effect profile associated with the existing anticancer therapy have necessitated the development of new therapeutic modalities to curb this disease. Bearing in mind the current scenario, a series of 1,2,3-triazole linked 3-(1,3-diphenyl-1H-pyrazol-4-yl)acrylates was synthesized following a multi-step reaction scheme. Initial screening for anticancer potential was done by in vitro sulforhodamine B assay against four human cancer cell lines- MCF-7 (breast), A549 (Lung) and HCT-116 and HT-29 (Colon). On evaluation, several compounds showed promising growth inhibition against all the cell lines, particularly compounds 6e, 6f and 6n. Among them, compound 6f displayed IC50 values of 1.962, 3.597, 1.764 and 4.496µM against A549, HCT-116, MCF-7 and HT-29 cell lines respectively. Furthermore, the apoptosis inducing potential of the compounds was determined by Hoechst staining and DNA fragmentation assay. Colony formation inhibition assay was also carried out to determine the long term cytotoxic potential of the molecules. Moreover, compounds 6e, 6f and 6n were also evaluated for anti-inflammatory activity by protein albumin denaturation assay and red blood cell membrane stabilizing assay.

1. Introduction

Cancer, a broad term used to characterize uncontrolled proliferation of cells arising from disruption or dysfunction of regulatory signalling pathways, which are normally under tight control, is one of the foremost diseases responsible for worldwide mortality [1]. At present, it is the major public concern due to the rapid growth of worldwide incidence. The major public concern has been the increase in deaths due to cancer. In developing and underdeveloped countries, lung, colon, breast and melanoma cancers are the most commonly reported cases [4]. However, emergence and spread of resistance to the currently available chemotherapeutic agents vindicates an urgent need for development of novel, more potent and selective anticancer agents [5].

Abbreviations: CuSO4·5H2O, Copper (II) sulphate pentahydrate; d, Doublet; DCM, Dichloromethane; DMF, N,N-Dimethyl formamide; DMSO, Dimethyl sulfoxide; ESI, Equipped with Electrospray; Hz, Hertz; IC50, Half Maximal Inhibitory Concentration; IR, Infra-red; J, Coupling Constant; m, Multiplet; NCI, National Cancer Institute; NMR, Nuclear Magnetic Resonance; NSAIDs, Non-Steroideal Anti-Inflammatory Drugs; PDB ID, Protein Data Bank ID; POCl3, Phosphorus oxychloride; ppm, Parts Per Million; p-TsOH, p-Toluene sulfonic acid; RCSB, Research Collaboratory for Structural Bioinformatics; s, Singlet; SP, Standard Precision; SRB, Sulforhodamine B; t, Triplet; TLC, Thin Layer Chromatography; TMS, Tetramethylsilane

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Link between chronic inflammation and cancer may be associated with different mechanisms like genomic instability induction, enhanced proliferation, inappropriate gene expression, resistance to apoptosis, metastasis, invasion through tumor-associated basement membrane, aggressive tumor neovascularization, etc. Elevated levels of reactive oxygen and nitrogen species can serve as major contributors in malignant cell transformation. Abnormal activation or overexpression of certain pro-inflammatory mediators like chemokines, cytokines, prostaglandins, cyclooxygenase-2, nitric oxide and inducible nitric oxide synthase promote tumor growth and progression. Certain pro-inflammatory mediators, particularly cytokines, chemokines and prostaglandins are known to endorse angiogenesis, thereby leading to metastasis and invasion [11].

Chronic inflammation plays a multifaceted role in carcinogenesis. Evidences from preclinical and clinical studies suggest that persistent inflammation functions as a driving force in the journey of cancer [12]. The inflammatory component contributes to tumor proliferation, angiogenesis, metastasis and resistance to hormonal and chemotherapy [13]. Chronic inflammation is also involved in the pathogenesis of insulin resistance, atherosclerosis, neurodegeneration and tumor growth [14]. Work reported by Aleksandrova et al. provides an evidence that elevated CRP concentrations (markers of inflammation) are related to a higher risk of colon cancer [15]. Except for few drugs, long term use of anti-inflammatory drugs including non-steroidal anti-inflammatory drugs (NSAIDs) results in adverse effects such as bleeding, gastrointestinal ulceration and kidney damage [16].

Pyrazole moiety can be traced in number of anticancer agents viz. Crizotinib, Tartrazine, Pyrazomycin, etc. [17]. This moiety is found in a number of anti-inflammatory drugs also. To mention, these are Celcoxib, Ramifinazone, Deracoxib, Lonozaic, etc. [18]. Similarly, triazole bearing compounds are also known to exhibit potent anticancer (Anastrazole, Letrozole, Vorozole) and anti-inflammatory (Carboxyamidotriazole) activities [19]. Several pyrazole [20,21] and triazole [22,23] bearing agents reported in literature possess dual activity i.e., anticancer and anti-inflammatory.

The diphenyl pyrazole moiety is receiving significant attention now a days, as the structural scaffold is found to make up the core structure of numerous anticancer (1) [24] and anti-inflammatory (2) [25] agents. Recent literature is enriched with progressive findings on anticancer (3) [26] and anti-inflammatory (4) [22] effects of 1,2,3-Triazole based compounds. Hybrids of diphenyl-pyrazole and 1,2,3-triazole were also reported as both anticancer (5 and 6) [27,28] and anti-inflammatory (7) [29] agents recently. Several studies also revealed the anticancer (8, 9) [30,31] and anti-inflammatory (10) [32] potential of acrylic acid derivatives, particularly the acrylates (Fig. 1).

Despite significant potency of acrylic linkage, literature search revealed that diphenyl pyrazole-4yl acrylic acid’s analogs are found to be rarely synthesized [33] and never found to be clubbed with 1,2,3-triazoles. Henceforth, the present work reports synthesis of novel 3-(1,3-diphenyl-1H-pyrazol-4-yl)acrylates linked with 1,2,3-Triazole (Fig. 2). The synthesized molecules were evaluated for their anticancer and anti-inflammatory activities. On evaluation, several compounds were found to be potent against both cancer and inflammation.

2. Results and discussion

2.1. Chemistry

Requisite compounds for Scheme 1 were obtained by employing multi-step reactions. Several substituted acetophenones were subjected to addition reaction with phenylhydrazine in the presence of glacial acetic acid. Subsequently, utilizing Vilsmeier-Haack reaction, formation of the pyrazole ring took place in the presence of N,N-dimethyl formamide (DMF) and phosphorus oxychloride (POCl3). DMF served as acylating agent while POCl3 acted as activating agent. Vilsmeier reagent, also known as Vilsmeier complex was formed during this reaction [34]. An aldol condensation’s modified form, Knoevenagel condensation reaction was used to convert pyrazole carbaldheydes into pyrazole carboxylic acids. In this, aldehyde derivatives were reacted with malonic acid in the presence of pyridine and piperidine. In this reaction, pyridine was used as the solvent whereas piperidine served as the catalyst. This reaction is based on nucleophilic addition between an active hydrogen compound (malonic acid in this case) and an aldehyde, resulting in the formation of α,β-unsaturated dicarboxylic acid [35]. Furthermore, monocarboxylic acid derivatives were obtained by decarboxylation of dicarboxylic acids via Doebner modification [36]. The acids were further reacted with propargyl bromide in the presence of potassium carbonate to yield terminal alkyne bearing pyrazole acrylate derivatives [37].

Aromatic azides were formed in high yields by aromatic amines via diazotization with sodium nitrite in water in the presence of p-toluene sulfonic acid (p-TsOH) and subsequent addition of sodium azide [38].

These synthesized terminal alkyne bearing pyrazole acrylates and aromatic azides were coupled in the final step in the presence of catalytic copper (II) sulphate pentahydrate (CuSO4·5H2O) and sodium ascorbate in a H2O/Dichloromethane (DCM) mixture at room temperature to yield the final compounds, triazoles [39].

2.2. Spectral characterisation

Spectral data for all the synthesized derivatives was found to be in concordance with the reported data and is discussed in detail in the Supporting Information section.

2.3. Biological activity

Anticancer potential was assessed of all the prepared compounds (4a-e, 6a-o). For assessing anticancer potential, the synthesized compounds were screened against a panel of four human cancer cell lines using the sulforhodamine B assay. Three of the most active compounds (6e, 6f and 6n) were further subjected to mechanistic studies including colony formation inhibition assay, Hoechst staining and DNA fragmentation assay.

Based on the reported facts that there exists a crucial link between cancer and inflammation. We also moved on the same aisle and got our most potent anticancer compounds (6e, 6f and 6n) evaluated for anti-inflammatory potential as well. Anti-inflammatory activity was performed using protein albumin denaturatation assay and red blood cell membrane stabilizing assay.

2.3.1. Anticancer

2.3.1.1. Sulforhodamine B assay.

The newly synthesized compounds (4a-e and 6a-o) were assessed for their in vitro cytotoxic potential against a panel of four human cancer cell lines i.e., MCF-7 (breast), A549 (Lung) and HCT-116 and HT-29 (Colon).

Initial anticancer screening was performed at concentration of 1 and 10µM (Table 1, Supplementary Information). The results of the preliminary anticancer screening were found to be in concordance with results of in silico studies. Half maximal inhibitory concentration (IC50) value was determined for the compounds showing more than 50% inhibition at a concentration of 10µM. Amongst the compounds of this series, compounds 6e, 6f and 6n were tested at concentrations of 1, 3, 5, 7 and 10 µM in order to determine IC50 values against the aforementioned panel of cell lines. On evaluation, compound 6f emerged as the most active compound with IC50 values of 1.962, 3.597, 1.764 and 4.496µM against A549, HCT-116, MCF-7 and HT-29 respectively. Another compound, 6n also displayed promising activity profile against HCT-116, MCF-7 and HT-29 with IC50 values of 4.940, 1.851 and 4.362µM respectively (Table 1). Depending on the results, colony formation inhibition assay, DNA fragmentation assay and fluorescence microscopy studies were also performed for compounds 6e, 6f and 6n.
2.3.1.2. Colony formation inhibition assay

Clonogenic cell survival assay is performed to assess the ability of a single cell to grow into a colony. It helps us in investigating the long term cytotoxic potential. Since the method reflects all modes of cell death or arrest, it is considered to be a standard for determining long term cell viability [40]. The colony formation of MCF-7 cells was found to be significantly inhibited by the exposure of MCF-7 cells to the compounds (Fig. 3). The clonogenic cell survival is found to be reduced approximately to its half.

(a) Diphenyl Pyrazole Containing Compounds

(b) 1,2,3-Triazole Containing Compounds

(c) Diphenyl Pyrazole and 1,2,3-Triazole Containing Compounds

(d) Acrylate Linkage Containing Compounds

Fig. 1. Anticancer and anti-inflammatory compounds bearing (a) diphenyl pyrazole, (b) 1,2,3-triazole, (c) diphenyl pyrazole and 1,2,3-triazole and (d) acrylate linkage.
and was found to be in agreement with IC_{50} values of the compounds. The result indicates that the molecules can efficiently inhibit proliferation and growth of MCF-7 cells.

2.3.1.4. DNA fragmentation assay. The higher order chromatin structure of DNA is degraded by the activated nucleases into mono and oligo nucleosomal DNA-fragments during the apoptotic process.

Table 1
IC_{50} values of compounds 6e, 6f and 6n against a panel of Human Cancer Cell Lines.

<table>
<thead>
<tr>
<th>Cell Line Type</th>
<th>A549</th>
<th>HCT-116</th>
<th>MCF-7</th>
<th>HT-29</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissue</td>
<td>Lung</td>
<td>Colon</td>
<td>Breast</td>
<td>Colon</td>
</tr>
<tr>
<td>S. No.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.</td>
<td>6e</td>
<td>10.607 ± 0.08</td>
<td>15.159 ± 0.34</td>
<td>10.184 ± 0.24</td>
</tr>
<tr>
<td>2.</td>
<td>6f</td>
<td>1.962 ± 0.16</td>
<td>3.597 ± 0.26</td>
<td>1.764 ± 0.18</td>
</tr>
<tr>
<td>3.</td>
<td>6n</td>
<td>9.463 ± 0.24</td>
<td>4.940 ± 0.45</td>
<td>1.851 ± 0.42</td>
</tr>
<tr>
<td>4.</td>
<td>Camptothecin</td>
<td>0.030 ± 0.17</td>
<td>0.050 ± 0.14</td>
<td>0.200 ± 0.24</td>
</tr>
<tr>
<td>5.</td>
<td>Combretastatin A4</td>
<td>0.180 ± 0.22</td>
<td>0.006 ± 0.06</td>
<td>0.033 ± 0.26</td>
</tr>
</tbody>
</table>
Therefore, a characteristic DNA ladder on agarose gel electrophoresis is formed as a result of loss of DNA content due to fragmentation of DNA [41]. Thus, in order to further confirm the apoptosis inducing effect of these compounds, DNA fragmentation assay was performed. MCF-7 cells were treated with the IC$_{50}$ concentration of compounds 6e, 6f and 6n for 24 h and chromosomal DNA was extracted. Agarose based gel electrophoresis was used to examine the apoptotic degradation of DNA. DNA from the cells treated by compounds exhibited a typical DNA smeared ladder pattern, an indicative of DNA fragmentation. In case of treatment with the synthesized compound 6f, a typical DNA smeared ladder pattern was observed that clearly indicated fragmentation (Fig. 5). DNA breaks at several positions across the chromosomal DNA resulted in smear of this type. Significantly intense smear was seen in DNA of cells treated with compounds 6f and 6n. However, moderate smear formation was seen in case of compound 6e. DNA from control cells showed little or no signs of DNA degradation. Results obtained using MCF-7 cells clearly indicate that compounds 6f and 6n are apoptosis inducing agents.

2.3.2. Anti-inflammatory

2.3.2.1. Protein albumin denaturation assay. Protein (egg albumin) denaturation assay was used to evaluate the in vitro anti-inflammatory activity of three most active compounds 6e, 6f and 6n. Diclofenac sodium was used as the standard drug for performing assay. All three of the compounds demonstrated significant anti-inflammatory activity with IC$_{50}$ values of 60.56, 57.24 and 69.15 µg/ml for compounds 6e, 6f and 6n respectively (Table 2). However, the IC$_{50}$ value for the standard, Diclofenac Sodium was found to be 54.65 µg/mL.

2.3.2.2. Red blood cell membrane stabilizing assay. In vitro anti-inflammatory activity was also assessed by another method called to be as red blood cell membrane stabilizing assay. Consequently, the results thus obtained revealed that the molecules inhibited RBC haemolysis in a concentration dependent manner (Table 3). Aspirin was used as standard. All of the compounds displayed noteworthy anti-inflammatory potential with compound 6f as the most promising one.

2.4. Molecular docking

In order to assess the mechanism of action of the synthesized novel anticancer derivatives, molecular docking studies were performed on different anticancer targets particularly, tubulin.

As a result of the molecular docking study, almost all of the
compounds have shown noteworthy and remarkable interactions with the tubulin protein. Among all the compounds used in the study, triazoles (6a-6o) were found to have better interactions than the terminal alkyne containing propargyl derivatives (4a-4e). The dock scores ranged from −8.047 to −5.442 kCal/mol, with 6f having the best score (−8.047) compared to the dock score of colchicine (−7.876). Table 4 represents the Dock Score of different synthesized compounds against tubulin protein.

Cartoon representation of the overall structure of the complex formed between 6f and tubulin is shown in Fig. 6a. α-tubulin is shown in grey (α1) and green (α2), whereas β-tubulin is represented by red (β1) and blue (β2) color. RB3 Stathmin-like domain is shown in yellow color. GTP (green) and GDP (orange) molecules bound to the α-tubulin and β-tubulin are displayed as CPK model. Also the most active compound (6f) can be seen bound to the β1-tubulin in purple color CPK model form.

In order to have a deeper look into the molecular interactions of the most active compound 6f and tubulin, the expanded image of the active site of β1-tubulin bearing compound 6f is displayed in Fig. 6b. Fig. 6c represent the Ligand Interaction Diagram (LID) depicting the molecular interactions of 6f with tubulin protein. To understand the molecular interactions displayed in LID more evidently, legends for the same are also represented below the diagram.

![DNA ladder assay of compounds 6e, 6f and 6n in MCF-7 cell line](image)

**Table 2**

<table>
<thead>
<tr>
<th>Comp.</th>
<th>% Inhibition</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>25 (µg/mL)</td>
<td>50 (µg/mL)</td>
</tr>
<tr>
<td>6e</td>
<td>64.99 ± 1.08</td>
<td>68.21 ± 0.67</td>
</tr>
<tr>
<td>6f</td>
<td>0.820 ± 0.69</td>
<td>74.43 ± 0.53</td>
</tr>
<tr>
<td>6n</td>
<td>19.51 ± 0.89</td>
<td>48.28 ± 0.59</td>
</tr>
<tr>
<td>Diclofenac Sodium</td>
<td>7.47 ± 0.66</td>
<td>67.31 ± 0.43</td>
</tr>
</tbody>
</table>

From the results, it is quite evident that compound 6f demonstrated interactions with a number of amino acid residues (Fig. 6c). The bridging Oxygen of the ester group was found to form a hydrogen bond with the amino acid residue Asn 258. Compound 6f interacted hydrophobically with numerous amino acid residues like Cys 241, Leu 242, Leu 248, Ala 250, Leu 252, Ala 316, Met 259, Pro 261, Trp 346, Ile 347, Val 355, etc. 6f also exhibited significant polar interactions with amino acid residues like Thr 239, Gln 247, Asn 249, Asn 258, Thr 314, Asn 349, etc. The compound was found to have interaction with positively charged residue Lys 352 as well. Sufficient amount of solvent exposure is also found to be contributing towards good affinity of the molecule with the protein.

**2.5. Structure-activity relationship**

On comparing the *in vitro* anticancer results of the synthesized molecules (4a-e, 6a-o), triazole containing compounds (6a-o) clearly demonstrated better results than the predecessor terminal alkyne containing acrylate derivatives (4a-e). The superior effect of di-phenyl pyrazole and triazole conjugates (6a-o) over di-phenyl pyrazole containing terminal alkynes (4a-e) may be attributed to the synergistic action of two pharmacophores when used in combination. Substitution of electron donating groups at –R position (over one of the phenyl ring containing terminal alkyne) demonstrated better results than the predecessor terminal alkyne containing acrylate derivatives (4a-e). The superior effect of di-phenyl pyrazole and triazole conjugates (6a-o) over di-phenyl pyrazole containing terminal alkynes (4a-e) may be attributed to the synergistic action of two pharmacophores when used in combination. Substitution of electron donating groups at –R position (over one of the phenyl ring containing acrylate derivatives (4a-e), triazole containing compounds (6a-o)) over di-phenyl pyrazole containing triazoles (6a-o) were found to possess poor anticancer potential.

In case of substitutions over –X position (over phenyl ring directly attached to the triazole ring), the situation was found to be just opposite. Here, substitution of electron withdrawing groups in 6f and 6n having methyl, methoxy and methoxy groups respectively. On the contrary, compounds bearing electron withdrawing groups like Chloro and Fluoro (4c, 4e, 6j and 6l) at position –R were found to have diminished anticancer activity. Unsubstituted compounds (6a, 6b, 6d, 6g and 6k) were found to possess mediocre anticancer potential.

**Table 3**

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Concentration (µg/mL)</th>
<th>% Protection</th>
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<tbody>
<tr>
<td>6e</td>
<td>100</td>
<td>40.25 ± 3.72</td>
</tr>
<tr>
<td>200</td>
<td>70.24 ± 1.02</td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>94.67 ± 1.84</td>
<td></td>
</tr>
<tr>
<td>6f</td>
<td>100</td>
<td>51.06 ± 1.09</td>
</tr>
<tr>
<td>200</td>
<td>74.25 ± 2.65</td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>97.25 ± 3.27</td>
<td></td>
</tr>
<tr>
<td>6n</td>
<td>100</td>
<td>46.24 ± 2.65</td>
</tr>
<tr>
<td>200</td>
<td>68.41 ± 3.01</td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>90.48 ± 2.08</td>
<td></td>
</tr>
<tr>
<td>Aspirin</td>
<td>100</td>
<td>60.14 ± 1.65</td>
</tr>
<tr>
<td>200</td>
<td>84.23 ± 2.95</td>
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</table>

**Fig. 5.** DNA ladder assay of compounds 6e, 6f and 6n in MCF-7 cell line, C is Control (Untreated) and M is Molecular Weight Marker.
3. Experimental

3.1. Chemistry

All the required chemicals and reagents used in the reactions were purchased from commercial vendors. These chemicals were not purified further. Aluminum backed silica plates (Merck, silica gel 60 F254) were used to perform Thin layer chromatography (TLC). Melting points were determined by UPLC (WATERS INC.). Perkin-Elmer-240 was used for recording Nuclear Magnetic Resonance (NMR) spectra. Frequencies of 400 and 100 MHz were used for recording 1H and 13C spectra respectively. CDCl3 served as the solvent to dissolve triazole derivatives whereas Tetramethylsilane (TMS) was taken as an internal standard. Chemical shift values (δ) are reported in parts per million (ppm) whereas coupling constants (J) are reported in Hertz (Hz). Xevo G2-XD QTOF, equipped with electrospray (ESI) ionization spray source was used to record high resolution mass spectra (HRMS). This was operated in full scan positive mode. For a few compounds, mass spectra were recorded on Synapt mass spectrometer (UPLCMS/MS). Purity of certain compounds was determined by UPLC (WATERS INC.).

3.1.1. Synthesis of 1,3-diphenyl-1H-pyrazole-4-carbaldehyde (2a-e)

1,3-Diphenyl-1H-pyrazole-4-carbaldehydes (2a-e) were synthesized as per the reported method in literature [42, 43]. The aldehydes thus obtained were found to have spectral and other analytical data in agreement with the data reported in the literature.

3.1.2. Synthesis of 3-(1,3-diphenyl-1H-pyrazol-4-yl)acrylic acid derivatives (3a-e)

Synthesis of 3-(1,3-diphenyl-1H-pyrazol-4-yl)acrylic acid derivatives was carried out according to our previously reported method. Details for derivatives 3a-e have already been published by our group [33]. Spectral details for newly synthesized derivatives 3d and 3e have been incorporated in the Supplementary Information section.

3.1.3. Synthesis of Prop-2-enyl-3-(1,3-diphenylpyrazol-4-yl)prop-2-enoate (4a-e)

Pyrazole acids (1 mM) (3a-e) were dissolved in dried acetone, and fused potassium carbonate (4 mM) was added to it. The resulting mixture was then heated with stirring for 30 min. After that, propargyl bromide (1 mM) was added and stirring with heating was continued till the completion of reaction. Potassium carbonate was then filtered from the reaction mixture, and the filtrate was poured in ice cold water. The precipitate thus obtained was filtered, washed with water and dried. Spectral details for compounds 4a-e are depicted in the Supplementary Information section.

3.1.4. Synthesis of aryl azides (5a-h)

Derivatives 5a-h were synthesized in accordance with the reported methods [38].

3.1.5. Synthesis of (1-phenyl-1H,1,2,3-triazol-4-yl)methyl 3-(1,3-diphenyl-1H-pyrazol-4-yl)acrylate (6a-6o)

Scheme 1 represents the method adopted for the preparation of triazoles reported in this paper. Different derivatives synthesized by this method are enlisted in Table 5. To a solution of terminal alkyne containing pyrazole acrylate derivatives (1 mM) (4a-e) and aryl azides (1.1 mM) (5a-h) in 1:1 mixture of water and DCM, sodium ascorbate (0.1 mM) and copper (II) sulphate (0.05 mM) were added sequentially. Reaction was stirred overnight at room temperature. Solvents were concentrated under vacuum and mixture was extracted with DCM, dried and concentrated under vacuum. Further, all the compounds were purified by column chromatography using silica gel and solvent system hexane and ethyl acetate in the ratio 7:3 v/v, respectively.

Table 4 Dock Score of Synthesized compounds 4a-4e and 5a-6o against tubulin protein (PDB ID: 3E22).

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</thead>
<tbody>
<tr>
<td>1.</td>
<td>4a</td>
<td>−6.611</td>
<td>8.</td>
<td>6c</td>
<td>−7.722</td>
<td>15.</td>
<td>6j</td>
<td>−7.192</td>
<td>2.</td>
<td>4b</td>
<td>−7.368</td>
</tr>
<tr>
<td>3.</td>
<td>4c</td>
<td>−6.290</td>
<td>10.</td>
<td>6e</td>
<td>−7.812</td>
<td>17.</td>
<td>6k</td>
<td>−7.708</td>
<td>4.</td>
<td>4d</td>
<td>−8.047</td>
</tr>
<tr>
<td>5.</td>
<td>4e</td>
<td>−6.116</td>
<td>12.</td>
<td>6g</td>
<td>−7.633</td>
<td>19.</td>
<td>6n</td>
<td>−7.377</td>
<td>6.</td>
<td>6a</td>
<td>−7.241</td>
</tr>
<tr>
<td>7.</td>
<td>6b</td>
<td>−7.377</td>
<td>14.</td>
<td>6i</td>
<td>−7.166</td>
<td>21.</td>
<td>6c</td>
<td>−8.376</td>
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3.1.5.1. (1-(2-nitrophenyl)-1H-1,2,3-triazol-4-yl)methyl 3-(1,3-diphenyl-1H-pyrazol-4-yl)acrylate (6a)

Compound 6a was synthesized by reaction of 4a with 5a as per the procedure given above. Appearance: White powder; Yield (%): 72; m.p. 176–178°C; 1H NMR (400 MHz, CDCl3): 5.41 (s, 2H, CH2), 6.28 (d, 1H, H-α, J = 16.0 Hz), 7.30 (t, 1H, H-4, J = 7.2 Hz), 7.43–7.51 (m, 5H, H-3, 3′, 4′, 5′, 5″), 7.61–7.81 (m, 8H, H-2,6,2′,6′,4″,5″,6″,β), 7.97 (s, 1H, H-Triazole), 8.09 (d, 2H, H-3″, J = 7.6 Hz), 8.25 (s, 1H, H-Pyrazole); IR (cm−1): 1715 (C=O), 1523 (NO2), 1239 (C=O); Mass (m/z): 493.2 [M + H]+; Elemental analysis (%) of C27H20N6O4, calculated/found: C (61.52/61.51), H (3.59/3.57), N (15.95/15.94).

3.1.5.2. (1-(3-nitrophenyl)-1H-1,2,3-triazol-4-yl)methyl 3-(1,3-diphenyl-1H-pyrazol-4-yl)acrylate (6b)

Compound 6b was synthesized by reaction of 4a with 5b as per the procedure given above. Appearance: White powder; Yield (%): 70; m.p. 196–198°C; 1H NMR (400 MHz, CDCl3): 5.44 (s, 2H, CH2), 6.28 (d, 1H, H-α, J = 16.0 Hz), 7.30 (t, 2H, H-4, J = 7.6 Hz), 7.42–7.51 (m, 5H, H-3, 3′, 4′, 5′, 5″), 7.66 (d, 2H, H-2″, J = 8.4 Hz), 7.74–7.82 (m, 4H, H-2′,6′,5″,β), 8.17 (dd, 1H, H-6″, J = 1.6 Hz), 8.20 (s, 1H, H-Triazole), 8.25 (s, 1H, H-Pyrazole), 8.31 (dd, 1H, H-4″, J = 8.8 & 1.6 Hz), 8.61 (t, 1H, H-2″, J = 2 Hz); IR (cm−1): 1719 (C=O), 1534, 1328 (NO2), 1219 (C=O); Mass (m/z): 493.5 [M + H]+; Elemental analysis (%) of C27H20N6O4, calculated/found: C (65.85/65.86), H (4.09/4.12), N (17.06/17.08).

3.1.5.3. (1-(2-nitrophenyl)-1H-1,2,3-triazol-4-yl)methyl 3-(3-(4-chlorophenyl)-1-phenyl-1H-pyrazol-4-yl)acrylate (6c)

Compound 6c was synthesized by reaction of 4e with 5a as per the procedure given above. Appearance: White powder; Yield (%): 70; m.p. 196–198°C; 1H NMR (400 MHz, CDCl3): 5.44 (s, 2H, CH2), 6.30 (d, 1H, H-α, J = 16.0 Hz), 7.34 (t, 1H, H-4, J = 7.6 Hz), 7.45–7.52 (m, 6H, H-3,3′,5′, 3′,4′,5′, 3′″,4′″,5′″), 7.60–7.74 (m, 3H, H-2,6″,4″), 7.72–7.76 (m, 4H, H-2″,6″,5″,β), 7.79 (t, 1H, H-5″, J = 8.8 Hz), 7.97 (s, 1H, H-Triazole), 8.10 (d, 1H, H-3″, J = 7.6 Hz), 8.24 (s, 1H, H-Pyrazole); IR (cm−1): 1721 (C=O), 1535, 1336 (NO2), 1267 (C=O); Mass (m/z): 527.7 [M + H]+; Elemental analysis (%) of C27H19ClN6O4, calculated/found: C (61.54/61.52), H (3.63/3.64), N (15.95/15.96).

3.1.5.4. (1-(3-chlorophenyl)-1H-1,2,3-triazol-4-yl)methyl 3-(1,3-diphenyl-1H-pyrazol-4-yl)acrylate (6d)

Compound 6d was synthesized by
Fig. 6. Docking analysis for most active anticancer compound 6f with tubulin (PDB ID: 3E22). (a) Cartoon representation of the overall structure of the complex formed between 6f and tubulin. (b) Dock pose of 6f bound in the colchicine binding site of β2-tubulin. (c) 2-D Ligand Interaction Diagram of 6f bound in the colchicine binding site of β2-tubulin.
reaction of 4a with 5c as per the procedure given above. Appearance: White powder; Yield (%): 69; m.p. 102–104°C; \(^1\)H NMR (400 MHz, CDCl\(_3\)): 5.44 (s, 2H, CH\(_2\)), 6.30 (d, 1H, H-α, J = 16.0 Hz), 7.33 (t, 1H, H-4, J = 7.6 Hz), 7.41–7.51 (m, 7H, H-3,5,′3′,5′,2″,4″), 7.58–7.64 (m, 2H, H-5,′5″), 7.66 (d, 2H, H-2, J = 6.8 Hz), 7.75 (d, 2H, H-2′,6″, J = 7.2 Hz), 7.77 (d, 1H, H-β, J = 15.6 Hz), 8.09 (s, 1H, H-1Traziole), 8.24 (s, 1H, H-Pyrazole); \(^1^3\)C NMR (100 MHz, CDCl\(_3\)): (57.48, 116.61, 117.50, 119.39, 125.98, 126.55, 127.29, 127.81, 129.76, 128.60, 128.72, 128.81, 129.59, 130.81, 133.14, 134.75, 136.21, 139.39, 142.90, 154.36, 166.84); IR (cm \(^{-1}\)): 1714 (C=O), 1256 (C–O); Mass (m/z): 482.3 [M + H]+; Elemental analysis (%) of C\(_2\)H\(_9\)FN\(_3\)O\(_2\) calculated/found: C (67.29/67.30), H (4.18/4.15), N (14.53/14.55).

3.1.5.5. (1-(3-chlorophenyl)-1H-1,2,3-triazol-4-yl)methyl 3-(3-(4-methoxyphenyl)-1-phenyl-1H-pyrazol-4-yl)acrylate (6c). Compound 6c was synthesized by reaction of 4d with 5c as per the procedure given above. Appearance: White powder; Yield (%): 74; m.p. 88–90°C; \(^1\)H NMR (400 MHz, CDCl\(_3\)): 3.87 (s, 3H, OCH\(_3\)), 5.44 (s, 2H, CH\(_2\)), 6.29 (d, 1H, H-α, J = 16.0 Hz), 7.00 (d, 2H, H-3′,5′, J = 8.4 Hz), 7.32 (t, 1H, H-4, J = 7.2 Hz), 7.45–7.52 (m, 4H, H-3,5,′3′,5′), 7.74–7.64 (m, 4H, H-16.5,′6″), 7.74 (d, 2H, H-2′,6″, J = 8.4 Hz), 7.80 (d, 1H, H-β, J = 16.0 Hz), 7.97 (d, 2H, H-2′,6″, J = 9.2 Hz), 8.20 (s, 1H, H-Traziole), 8.24 (s, 1H, H-Pyrazole), 8.41 (d, 2H, H-3′,5″, J = 9.2 Hz); IR (cm \(^{-1}\)): 1722 (C=O), 1263 (C–O); Mass (m/z): 512.6 [M + H]+; Elemental analysis (%) of C\(_2\)H\(_9\)FN\(_3\)O\(_2\) calculated/found: C (65.69/65.66), H (4.33/4.35), N (13.68/13.69).

3.1.5.6. (1-(4-nitrophenyl)-1H-1,2,3-triazol-4-yl)methyl 3-(1-phenyl-3-(3-p-toly1)-1H-pyrazol-4-yl)acrylate (6f). Compound 6f was synthesized by reaction of 4b with 5d as per the procedure given above. Appearance: White powder; Yield (%): 75; m.p. 194–196°C; \(^1\)H NMR (400 MHz, CDCl\(_3\)): 2.42 (s, 3H, CH\(_3\)), 5.43 (s, 2H, CH\(_2\)), 6.26 (d, 1H, H-α, J = 16.0 Hz), 7.34 (t, 1H, H-4, J = 7.2 Hz), 7.44–7.52 (m, 4H, H-3,5,′3′,5′), 7.65 (d, 2H, H-2, J = 6.8 Hz), 7.75 (d, 2H, H-2′,6″, J = 8.0 Hz), 8.10 (s, 1H, H-Traziole), 8.22 (s, 1H, H-Pyrazole); IR (cm \(^{-1}\)): 1722 (C=O), 1263 (C–O); Mass (m/z): 507.2 [M + H]+; Elemental analysis (%) of C\(_2\)H\(_9\)FN\(_3\)O\(_2\) calculated/found: C (66.40/66.43), H (4.38/4.36), N (16.59/16.60).

3.1.5.7. (1-(4-fluorophenyl)-1H-1,2,3-triazol-4-yl)methyl 3-(1-diphenyl-1H-pyrazol-4-yl)acrylate (6g). Compound 6g was synthesized by reaction of 4a with 5e as per the procedure given above. Appearance: White powder; Yield (%): 67; m.p. 150–152°C; \(^1\)H NMR (400 MHz, CDCl\(_3\)): 5.41 (s, 2H, CH\(_2\)), 6.28 (d, 1H, H-α, J = 16.0 Hz), 7.20 (t, 2H, H-3″,5″, J = 8.8 Hz), 7.33 (t, 1H, 4, J = 7.2 Hz), 7.42–7.52 (m, 5H, H-3,5,′3′,5′, 7.66 (d, 2H, H-2, J = 6.8 Hz), 7.70–7.73 (m, 2H, H-2″,6″), 7.75 (d, 2H, H-2′,6″, J = 6.4 Hz), 7.77 (d, 1H, H-β, J = 15.6 Hz), 8.04 (s, 1H, H-Traziole), 8.24 (s, 1H, H-Pyrazole); \(^1^3\)C NMR (100 MHz, CDCl\(_3\)): (57.47, 116.49, 116.67, 116.90, 117.45, 119.40, 122.43, 122.59, 122.68, 125.55, 127.33, 127.83, 128.82, 129.60, 132.13, 136.32, 139.38, 143.95, 153.48, 166.93); IR (cm \(^{-1}\)): 1714 (C=O), 1219 (C–O); Mass (m/z): 511.6 [M + H]+; Elemental analysis (%) of C\(_2\)H\(_9\)FN\(_3\)O\(_2\) calculated/found: C (63.53/63.55), H(3.75/3.77), N(16.46/16.43).

3.1.5.11. (1-(3-chloro-4-fluorophenyl)-1H-1,2,3-triazol-4-yl)methyl 3-(1-diphenyl-1H-pyrazol-4-yl)acrylate (6k). Compound 6k was synthesized by reaction of 4a with 5g as per the procedure given above. Appearance: Brick red powder; Yield (%): 74; m.p. 160–162°C; \(^1\)H NMR (400 MHz, CDCl\(_3\)): 5.41 (s, 2H, CH\(_2\)), 6.28 (d, 1H, H-α, J = 16.0 Hz), 7.29–7.37 (m, 2H, H-4,5′), 7.44–7.51 (m, 5H, H-
3.5.3′,4′,5′), 7.61–7.67 (m, 3H, J = 6.8 Hz), 7.77 (d, 1H, H-β), J = 16.0 Hz), 7.84 (d, 1H, H-6′, J = 6.4 Hz & 2.4 Hz), 8.05 (s, 1H, H-Triazole), 8.24 (s, 1H, H-Pyrazole); IR (cm⁻¹): 1720 (C=O); 1230 (C=O); Mass (m/z): 500.1 [M + H]^+; Elemental analysis (%) of C_{27}H_{19}ClFN_{5}O_{2}, calculated/found: C (67.81/67.82), H (4.47/4.45), N (13.22/13.24).

3.1.5.12. (1-o-tolyl)-1H-1,2,3-triazol-4-yl)methyl 3-(3-(4-chlorophenyl)-1-phenyl-1H-pyrazol-4-yl)acrylate (6f). Compound 6f was synthesized by reaction of 4e with 5f as per the procedure given above. Appearance: White powder; Yield (%): 80; m.p. 208–210°C; 1H NMR (400MHz, CDCl_3): 2.42 (s, 6H, 2×CH_3), 5.41 (s, 2H, CH_2), 6.27 (d, 1H, H-3′, J = 8.8 Hz); 7.15 (d, 1H, H-5′, J = 8.8 Hz); 7.32 (t, 1H, H-4′, J = 6.8 Hz); 7.46–7.60 (m, 6H, H-2,3,5,6,2′,6′); 7.74–7.79 (m, 3H, H-2′,6′,β); 8.00 (s, 1H, H-Triazole); 8.21 (s, 1H, H-Pyrazole); IR (cm⁻¹): 1725 (C=O), 1238 (C=O); Mass (m/z): 496.4 [M + H]^+; Elemental analysis (%) of C_{28}H_{22}ClN_{5}O_{2}, calculated/found: C (65.85/65.83), H (4.11/4.10), N (17.06/17.07).

3.2. Biological activity

3.2.1. Anticancer

3.2.1.1. Sulforhodamine B assay. For evaluating anticancer potential of the synthesized compounds, certain human cancer cell lines- MCF-7 (breast), A549 (Lung) and HCT-116 and HT-29 (Colon) were obtained from National Cancer Institute (NCI). Growth of all these cell lines was achieved in tissue culture flasks in complete medium (RPMI-1640) supplemented with100μg/mL streptomycin, 10% fetal bovine serum and 100 units/mL penicillin in carbon dioxide incubator (New Brunswick, Galaxy 170R, Eppendorf) at 5% CO_2, 37°C and 98% RH.

96-well cell culture plates were employed for performing sulforhodamine B (SRB) assay. To each well, 100μL of the cell suspension of desired density was added and incubated (5% CO_2, 37°C, 90% RH) for 24h. Following incubation for the stipulated time period, 100μL of the test samples were added into the wells supplemented with cells. These culture plates were again incubated for a period of 48h. Depending on the requirements, controls, blanks and positive controls were also included in every experiment. Following incubation for the stipulated period, cells were fixed using 50μL of ice-cold TCA (50%) for 1 h at a temperature of 4°C. Further, plates were washed using distilled water and then air dried. Following complete drying, addition of SRB dye (100μL) was made to every well and was then allowed to stain for 30 min at room temperature. In order to remove excessive dye, plates were washed with 1% acetic acid for five times. These plates were then air dried. Solubilisation of adsorbed dye was done by addition of 100μL of 10 mM Tris Buffer (pH 10.5) to each well. Following this, these plates were shaken on shaker platform for a duration of 5 min. 96-well ELISA plate reader (Molecular Devices, Sunnydale, USA) was used for reading these plates. Every sample was run in triplicate fashion. Mean values of these readings was taken for getting the results. Concentration of DMSO in cultures was kept < 1% [44].

\% Cell Viability = 100 × (T − T_0)/(C − T_0)

\% Growth inhibition = 100-%Cell viability.

\[ T: \text{Absorbance of Test sample} \]
\[ T_0: \text{Absorbance of Blank} \]
\[ C: \text{Absorbance of Control} \]

3.2.1.2. Colony formation inhibition assay. Plating of MCF-7 cells at a density of 500 cells/well was done into 6-well culture plates. An adherence period of 24h was given before treatment. Incubation of the cells was done with culture medium containing compounds 6e, 6f and 6n at IC_{50} concentration. Following a period of 24h, medium was replaced with fresh medium and further, the cells were incubated for a period of 14 days. Washing of cells was then done using 150mM PBS (pH 7.4) followed by fixing using 4% paraformaldehyde, staining with 0.5% methylene blue in 10% ethanol for 30 min and finally rinsing with distilled water for removing excess dye. Plates were then photographed using a digital camera.
cells were subjected to the treatment with IC50 concentration of compounds 6e, 6f and 6n for 24 h. Trypsinisation was used to harvest the cells and centrifugation at 2500 rpm for 5 min at 4°C was done. Then the pellet was collected and washing was done using phosphate buffered saline (150 mM PBS; pH 7.4). 400 µg/mL DNsase free RNase A containing 250 µL of lysis buffer (100 mM NaCl, 5 mM EDTA, 10 mM Tris HCl pH 8.0, 0.25% SDS) was added and incubated for 90 min at 37°C. This was followed by incubation with proteinase K (200 µg/mL) for 1 h at 50°C. The samples were then centrifuged for 5 min at 3000 rpm at 4°C and the supernatant was then collected. 65 µL of ammonium acetate (10 M) and ice cold ethanol (500 µL) was added and mixed well. The samples were then incubated for 1 h at −80°C and were further subjected to centrifugation at 12000 rpm at 4°C for 20 min. Washing of the pellet with 80% ethanol was done followed by air-drying for 10 min at room temperature. After drying, the pellet was dissolved in 50 µL of TBE buffer. Finally, 1.5% agarose gel electrophoresis in TBE Buffer was used to visualize DNA laddering after staining with ethidium bromide, which was followed by photography using digital camera.

3.2.2. Anti-inflammatory

3.2.2.1. Protein albumin denaturation assay. In vitro protein denaturation method was adopted for determining anti-inflammatory potential of the most active anticancer compounds 6e, 6f and 6n. Method described by Mizushima and Kobayashi was used for in vitro assessment. Bovine serum albumin and egg albumins were dissolved in 50 mM sodium phosphate buffer (pH 6.4) at a concentration of 1%. The reaction mixture comprised test sample (0.1 mL, 1 mg/mL) and 0.2 mL albumin protein. Final volume of the reaction mixture was made to 5 mL using buffer. Incubation of the reaction mixture was done at 37°C for a period of 20 min. Following this, the reaction mixture was brought to room temperature and turbidity was measured using UV–visible spectrophotometer at 660 nm. The entire experiment was done in triplicate and average values were calculated. Finally, percentage inhibition of protein denaturation was calculated using the following formula.

\[ \text{Percent Inhibition} = \left( \frac{\text{Abs. Control} - \text{Abs. Sample}}{\text{Abs. Control}} \right) \times 100 \]

Here:
Abs. Control: Absorbance of Control
Abs. Sample: Absorbance of Test sample

3.2.2.2. Red blood cell membrane stabilizing assay. In this method, 5 mL of blood was collected from healthy adult rat and mixed with 5.0 mL of Alsever solution. Alsever solution comprised of 2.0% dextrose, 0.8% sodium citrate, 0.5% citric acid and 0.42% NaCl in water. The complete mixture was then centrifuged at 300 rpm. Packed cells were washed with isosainole (0.85%, pH 7.2) thrice for removal of cell debris. Assay mixture comprised of different concentrations of test sample (100, 200 and 500 µg/mL), 1.0 mL phosphate buffer (0.15 M, pH 7.4), 2.0 mL hyposaline (36%) and 0.5 mL red blood cell suspension. The complete mixture was incubated at 37°C for 30 min, followed by centrifugation at 3000 rpm for 20 min. Spectrophotometer was used for determining haemoglobin content of the supernatant at 560 nm. Aspirin (100 and 200 µg/mL) served as the standard. Percentage of RBC membrane stabilization was determined using formula.

\[ \% \text{Protection} = \left( \frac{\text{OD}_1 - \text{OD}_2}{\text{OD}_1} \right) \times 100 \]

Here:
\( \text{OD}_1 \): Optical density of test sample
\( \text{OD}_2 \): Optical density of control

3.3. Molecular docking

Catalytic domain of tubulin with PDB ID 3E22 was downloaded from RCSBP protein data bank. Protein preparation was done using protein preparation wizard, a module of Schrödinger 2016-1. Water molecules without 3H bonds were removed. Following this, addition of hydrogen bonds corresponding to pH 7 was made considering proper ionization states for both basic and acidic amino acid residues. The energy of the crystal structure was minimized by using force field, OPLS-2005. Further, colchicine bound to tubulin protein was selected as its center for generating the grid box at active site’s centroid. Around the centroid, a radius of 16 Å was selected in order to define the active site.

Finally, docking of all low energy conformations into the catalytic domain of protein was done using Grid based Ligand Docking with Energetics (Glide v7.0, Schrödinger 2016-1) in standard precision (SP) mode in the absence of any constraints.

4. Conclusion

In the present study, synthesis and anticancer evaluation of series of 1,2,3-triazole linked 3-(1,3-diphenyl-1H-pyrazol-4-yl)acrylates have been carried out. Amongst all the synthesized compounds, compound 6f showed most promising anticancer effects with IC50 value of 1.962, 3.597, 1.764 and 4.496 µM against A549, HCT-116, MCF-7 and HT-29 cell lines respectively. Further, studies like Hoechst staining and DNA fragmentation assay strengthened the preliminary results and ascertained the role of apoptosis induction as the mechanism of action of the synthesized compounds. Anti-inflammatory assessment for the three most potent anticancer compounds was performed by protein albumin denaturation assay and red blood cell membrane stabilizing assay. On the whole, it was observed that all the synthesized compounds demonstrated significant anti-inflammatory potential in addition to anticancer effects.

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Conflict of interest

Authors declare no conflict of interest.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2019.03.071.

References
