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ORIGINAL ARTICLE

Synthesis of Novel Quinazolines and its Application as Biomarkers for Apoptotic Cells

Atulkumar A Kamble[1], Barnabas Kodasi[2], Sandhya Kumari[3], Guruprasad Kalthur[3], Praveen K Bayannavar[2], Ravindra R Kamble[1,*]

[1]*Department of Chemistry, K. L. E. Society's G. I. Bagewadi's Arts Science and Commerce College, Nipani, 591237, Karnataka, India*
[2]*Department of Studies in Chemistry, Karnataka University, Dharwad, 580003, Karnataka, India*
[3]*Department of Clinical Embryology, Kasturba Medical College, Manipal, Manipal Academy of Higher Education, Manipal, 576104, Karnataka, India*

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* *Corresponding author.*
Ravindra R Kamble
ravichem@kud.ac.in

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ABSTRACT

The current study provides a straightforward, simple, and efficient protocol for the microwave- assisted synthesis of a series of 2,3-dihydroquinazolinones and its affinity to bind to DNA so as to exhibit potential anticancer activity. The predominance of this method is smooth synthetic pathway, transient reaction times, facile workup, and exceptional yields. Docking studies manifested strong binding interactions with BSA enzyme (PDB ID: 3V03). The compound **6d** was exceptional in binding to DNA. Compound **6d** showed significant cytotoxicity and genotoxicity against *Ehrlich Ascites Carcinoma* (EAC) cells. UV–Vis absorption and Fluorescence studies were carried out for compound **6d** showing promising results.

Keywords: 2,3-dihydroquinazolinones; Cytotoxicity; Genotoxicity; UV–Vis absorption studies; Fluorescence studies

1 INTRODUCTION

2,3-Dihydro-quinazolin-4(1*H*)-ones have been found in several important natural and synthetic organic molecules. They have been accepted as a useful privileged scaffold in the design and synthesis of library of compounds and in drug discovery applications [1]. Many 2,3-dihydroquinazolinone derivatives are reported to be possessing interesting biological agents such as anticancer [2], anti-inflammatory [3], anticonvulsant [4] and antidepressant [5] etc. Most of the 2,3-dihydro-quinazolin-4(1*H*)-one derivatives were also used as anticancer agents and have shown phototoxic activity. Figure 1 depicts some quinazolines moieties have clinical applications.

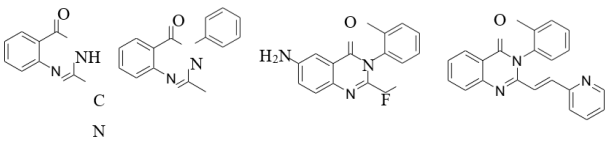

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Figure 1: Some

In view of exhibition of useful pharmacological properties, a number of synthetic routes have been reported for the synthesis of 2,3-dihydroquinazolinones. For ex: synthetic methods based on cyclization of aminobenzamide derivatives with substituted benzaldehydes in presence of ionic liquids [6], ammonium chloride [7], tetrabutyl ammonium bromide [8], cerium (IV) ammonium nitrate (CAN) [9], amberlyst-15 [10], β- cyclodextrin [11], $TiCl_4$/Zn [12], Bronsted and phosphoric acids [13, 14], $CuCl_2$ [15], *p*TSA [16], etc. have been used. However, some of these procedures have certain limitations such as time consuming, harsh conditions, tedious process, high temperatures, expensive reagents and low yields. Thus, the development of

novel methods for the synthesis of 2,3-dihydroquinazolin-4(1*H*)-ones is of great importance because of their potential biological activities.

The detailed synthetic aspects and their biological significance of 2,3- dihydroquinazolin-4(1*H*)-ones were discussed in the earlier report [17].

Similarly, heterocyclic compounds containing pyrazole nucleus are well-known and have pharmacological relevance [18, 19]. The pyrazole ring can be traced in a number of well-known drugs belonging to different categories with diverse therapeutic activities. Pyrazole has been considered as admired scaffold because of its various pharmacological activities like antitubercular [20, 21], anti-inflammatory [22, 23], analgesic [24], antimicrobial [25, 26], anticancer [27, 28], anticonvulsant [29, 30], cardiovascular [31] etc. Owing to the diverse pharmacological activities of the pyrazole derivatives, a number of researchers are engaged in the development of pharmacologically active drugs bearing pyrazole moiety. Some of the clinically active drugs (**V-VIII**) containing pyrazole ring are shown in Figure 2 [32].

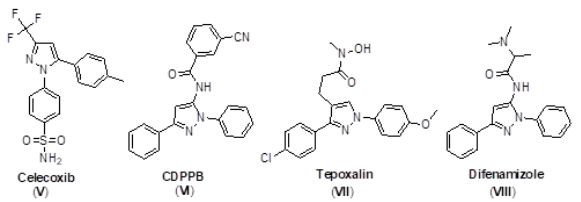


Figure 2: Pyrazole containing pharmacological drugs

DNA is referred as the molecule of heredity and it is responsible for the genetic propagation of all living bodies [33, 34]. From several years, it has been a considerable interest in DNA binding properties with different organic small molecules. Many such molecules have been used as tools for understanding DNA structure. DNA is an important cellular receptor; different molecules have shown antitumor activity by binding to DNA and changes the replication of DNA and inhibits the growth of the tumor cells. Moreover, the efficacy of such molecules depends on the mode of binding ability to the DNA strands [35]. Therefore, understanding the interaction between drugs and DNA plays a key role in drug designing, pharmacology and is of great significance for the synthesis of new drugs targeted to DNA [36].

Similarly, proteins are the most abundant macromolecules in cells and are crucial maintaining normal cell functions. Bovine serum albumin (BSA) is one of the major components in plasma protein, plays an important role in transporting and metabolizing many endogenous and exogenous compounds in metabolism [37]. Binding of the organic molecules with proteins is an important factor in the study of pharmacokinetics and pharmacodynamics of the molecules [38]. The investigation of drug-protein interaction is essential for an understanding of the drug action mechanism and in designing new drugs [39, 40].

DAPI [2-(4-amidinophenyl)-1*H*-indole-6-carboxamidine] is a fluorescent dye which exhibits several binding modes to DNA [41] and it has been widely utilized as a DNA specific probe for flow cytometry, chromosome staining, DNA visualization and quantitation [42] and has become now an important tool in molecular biology. Based on the above facts herein, we report synthesis of 2-(1*H*-pyrazol-4-yl)-quinazolin-4(1*H*)-ones **6a-d** using anhydrous K_2CO_3 as efficient and ecofriendly catalyst in shorter reaction time under mild reaction condition in microwave reactor. Further, their cytotoxicity, genotoxicity and DNA/protein interactions have been investigated in this chapter in hope of showing the properties like DAPI.

2 MATERIALS AND METHODS

All the required chemicals used were of analytical grade and purchased from commercially available suppliers and were used without purification. Thin layer chromatography (TLC) was performed using 0.20 mm Aluchrosep silica gel 60 F254 plates (SD. Fine, Mumbai) and visualized under UV chamber for monitoring the progress of the reaction. Melting point of novel compound was recorded on Coslab melting point apparatus. The FT-Infrared spectra were recorded in the region 4000- 500 cm^{-1} on a PerkinElmer Spectrum Version 10.5.4. ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded on a Bruker Avance FT-NMR spectrometer using CDCl3 as the solvent and with tetramethylsilane (TMS) as an internal standard. All the chemical shift values were reported in δ scale downfield from TMS and J values were given in Hz. The mass spectra were recorded on Shimadzu GC-MS operating at 70 eV. Elemental analyses for C, H and N were examined using a Heraeus CHN rapid analyzer.

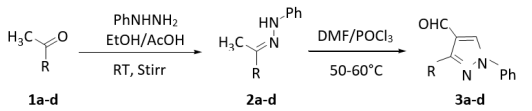
3 RESULTS AND DISCUSSION

Microwave-assisted synthesis is a technique of green chemistry, and which has gained much attention in recent years. Microwave irradiation-assisted chemical transformations are eco-friendly, pollution free, offer high yields and easier in processing and handling [43–45].

Reactions carried out conventionally, such as heating on oil baths, sand baths and mantles, is not only slow but it creates a hot surface on the reaction vessel where the products, substrates and reagents often decomposes. In microwave reactions, energy is introduced into the chemical reactor remotely and passes through the walls of the reaction vessel, heating the reactants and solvents directly. The use of microwave energy instead of conventional heating often results in good yields in a short time as compared to the reaction by classical synthetic methods [46–48]. Nowadays, microwave-assisted organic synthesis has gained widespread importance in drug discovery. Microwave synthesis, by

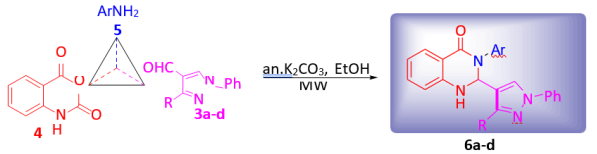
accelerating chemical reactions from hours or days to minutes, provides quick results. It enables synthesis that was not previously possible by classical methods [49].

In this work, we investigated a simple and efficient protocol for the microwave- assisted synthesis of a series of 2,3-dihydroquinazolinones with affinity to bind to DNA so as to exhibit potential anticancer activity.



a: R = *p*-Cl-C_6H_4, **b**: R = *m*-Br-C_6H_4, **c**: R = *p*-NO_2-C_6H_4, **d**: R = -C_5H_4N (pyridin-3-yl)

Figure 3: Scheme 1. Synthesis of 1-phenyl-3-substituted-1*H*-pyrazole-4-carbaldehydes 3a-g



a: R = *p*-Cl-C_6H_4, **b**: R = *m*-Br-C_6H_4, **c**: R = *p*-NO_2-C_6H_4, **d**: R = -C_5H_4N (pyridin-3-yl)

Figure 4: Scheme

Synthesis of 1-phenyl-3-substituted aryl/heteroaryl-1*H*-pyrazole-4-carbaldehydes **3a-d** and 2-(3-aryl/heteroaryl)-1-phenyl-1*H*-pyrazol-4-yl)-2,3-dihydro-3-phenylquinazolin-4(1*H*)-ones derivatives **6a–d** is outlined in **Scheme 1** and **2**. In **Scheme 1** condensation of aryl/heteroaryl acetophenones **1a-d** and phenyl hydrazine gave corresponding Schiff bases which followed formylation via Vilsmeier-Haak reaction to give 1-phenyl-3-substituted-1*H*-pyrazole-4-carbaldehydes **3a-d**. In **Scheme 2,** one pot synthesis of 2-(3-(aryl/heteroaryl)-1-phenyl-1*H*-pyrazol-4-yl)-2,3-dihydro-3- phenylquinazolin-4(1*H*)-ones derivatives **6a–d** was achieved by microwave irradiation of mixture of isatoic anhydride **4**, aniline **5** and **3a-d**. All the final products were purified by column chromatography using hexane and ethyl acetate as an eluent.

3.1 Spectral characterization

IR spectral analysis of the compounds **6a-d** showed -NH stretching frequency as a broad medium intensity band at 3302-3364 cm^{-1}. The carbonyl (C=O) stretching frequency has appeared in the range 1633-1658 cm^{-1} of the quinazoline ring.

^{1}H NMR spectral analysis of the compounds **6a-d** exhibited one singlet in the range 10.02-10.08 ppm due to -NH proton of quinazolinone ring and another singlet in the range 8.05-8.52 ppm appeared due to pyrazole C_5-H proton. Quinazolinone C_2-H proton appeared as singlet in the range 4.63-4.72 ppm. The remaining aromatic protons have appeared in their respective region. Similarly, ^{13}C NMR spectral analysis showed the number of carbon peaks in accordance with the number of magnetically non-equivalent carbons present in the molecule. Mass spectral analyses showed the molecular ion peaks equivalent to their respective molecular masses (please see Supporting Information).

3.2 Molecular docking studies

Molecular docking plays an important role in the rational drug design and used to predict the bonding affinity and binding energies of the small molecule drug candidates to the active site of their targets [50]. Molecular docking was performed with Surflex-Dock program that is interfaced with Sybyl-X 2.0. [51]. Crystal structure of Bovine Serum Albumin was collected from PDB under code 3V03 [52] and was extracted from the Brookhaven Protein Database (PDB: http://www.rcsb.org/pdb). All the hydrogen atoms were added to define the correct configuration and tautomeric states. Then the modelled structure was energy-minimized using Tripos force field with distance dependent dielectric function and partial atomic charges were calculated by AMBER7F9902 method and finally water molecules were removed from the model. The geometry of the molecule CP was subsequently optimized to minimal energy using the Powell energy minimization algorithm, Tripos force field with Gasteiger-Hückel charges. The CP was then separately docked into the binding pocket for docking-scoring analysis. To identify the ligand-protein interactions, the top pose and protein were loaded into work area and the MOLCAD (Molecular Computer Aided Design) program was employed to visualize the binding mode between the protein and ligand.

3.3 Protein docking stimulation

Surflex-docking was employed to understand the interaction between BSA and the synthesized compounds and to ultimately elucidate the interaction mechanism. All the 4 inhibitors were docked into the active site of ENR as shown in Figure 5. The predicted binding energies of the compounds are listed in **Table A** (please see Supporting Information). As depicted in the Figure 6. Compound **6b** forms a hydrogen bonding interaction at the active site of the enzyme (PDB ID: 3V03), nitrogen atom of pyrazole ring makes hydrogen interaction with hydrogen of LYS431 (-N —— H-LYS431, 2.60 Å).

It can be observed in **Figure A** (please see Supporting Information), that compound **6d** makes two hydrogen bonding interactions at the active site of the enzyme (PDB ID: 3V03), nitrogen atom of pyridine ring makes two hydrogen interactions with hydrogen of ARG185 (-N —— H-ARG185, 2.67 Å; -N H-ARG185, 2.72 Å). **Figure B** (please see Supporting Information) shows the hydrophobic and hydrophilic amino acids surrounded to the compounds **6b** and **6d**.

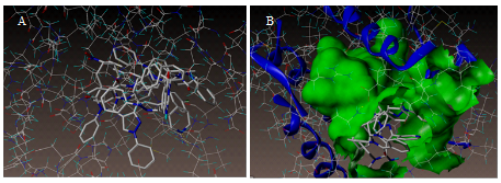


Figure 5: Docked view of all the compounds at the active site of the enzyme PDB ID: 3V03

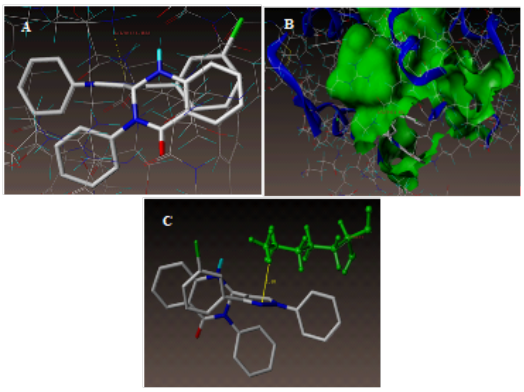


Figure 6: Docked view of compound 6b at the active site of the enzyme PDB: 3V03

3.4 *Pharmacology*

3.4.1. *Fluorescence screening of compounds 6a-d*

Fluorescence is the phenomenon of emission of light by a substance when it absorbed light. Generally, the emitted light by the substance has a longer wavelength, and therefore lower energy, than the absorbed radiation. Fluorescence occurs when the absorbed radiation is in the ultraviolet region and thus invisible to the human eye, while the emitted light is in the visible region, which gives the fluorescent substance a distinct color that can only be seen when exposed to UV light. Now a days, organic chromophores have been attracting much attention for their wide applications in data storage [53], sensor [54], photo switch [55], organic light-emitting diode (OLED) [56], fluorescent biological markers [57] etc. Therefore, in the present chapter we have investigated the fluorescence property of synthesized 2,3-dihydroquinazolinone derivatives **6a-d**.

Ehrlich Ascites Carcinoma (EAC) cells were incubated with **6a, 6b, 6c** and **6d** and scanned under fluorescent microscope using different filters. Compound **6b** had signal but it was quenching fast whereas, compounds **6a** and **6c** did not show any fluorescence signal under any of the filter used. Interestingly, compound **6d** had good stable signal when observed under fluorescent microscope which was further confirmed using confocal microscopy (405 nm laser excitation and collecting 450 nm emission) at 100 μg/mL concentration. As shown in Figure 7 it is clearly evident that the compound **6d** binds to DNA. **Figure C** (please see Supporting Information) depicts comparative assessment of cell death in *EAC* cells by tryphan blue method and fluorescence method using **6d** compound under DAPI filter.

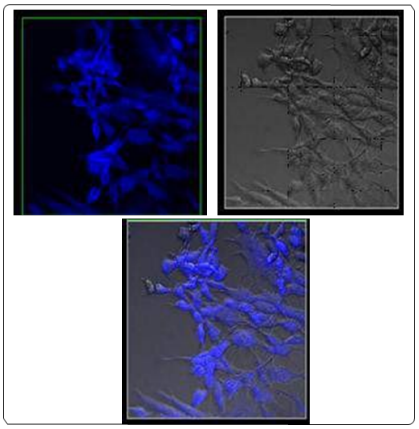
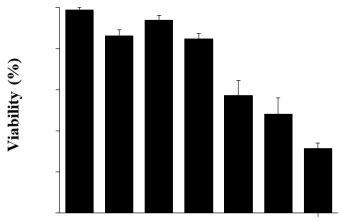


Figure 7: Images of Apoptotic cells stained with compound 6d

3.4.2. *Cytotoxic and genotoxic effect of Ehrlich Ascites Carcinoma (EAC) cells with compound 6d*

Since most of the dyes which bind to DNA exhibit DNA damaging effect, the compound **6d** was further evaluated for cytotoxicity and genotoxicity. The stock solution of compound **6d** was prepared by dissolving compound **6d** (0.01 g) in DMSO (1.0 ml). From this stock solution working solutions of various concentrations (10, 25, 50, 100 and 200 μg/mL) were prepared for each compound.

At lower concentration (up to 25 μg/mL) this compound did not have any significant cytotoxic effect as indicated by cell viability data (Figure 8). At 50 μg/mL concentration almost 50% reduction in viability was observed which further decreased with increase in concentration of **6d** ($p< 0.001$ compared to control) (Table 1).



[a]P<0.001vs Control; [b]P<0.01, [c]P<0.001 vs VC; [d]P<0.001 vs 10µg/mL; [e]P<0.01 vs 25µg/mL; [f]P<0.001 vs 25µg/mL; [g]P<0.05 vs 50µg/mL

Figure 8: Effect of compound 6d onthe viability of *EAC* cells at 24h after culture assessed by tryphan blue dye exclusion method

The control cells had 2 % of micronucleated cells which was marginally higher in vehicle control. Even though at 10 μg/mL concentration of compound **6d** there was almost two times higher percentage of micronucleated cells, the

difference was statistically not significant (Figure 9). With further increase in 25, 50 and 100 μg/mL concentration, a significant increase in micronucleated cells was observed ($p<0.001$). However, at highest concentration, the percentage of micronucleated cells decreased compared to the lower doses, which is probably due to the low cell proliferation and high cell death observed at this concentration (Figure 10).

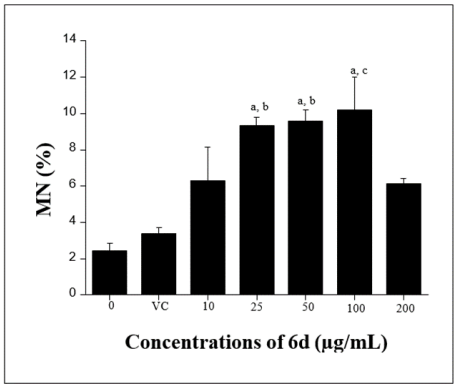


Figure 9: Effect

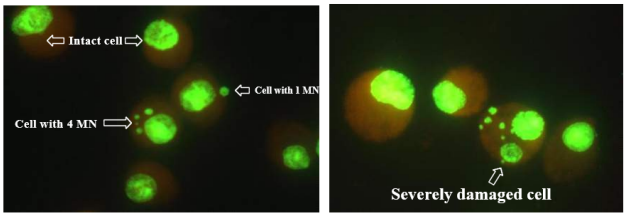


Figure 10: *Ehrlich Ascites Carcinoma* cells stained with acridine orange to assess the genotoxicity by micronucleus assay. Representative images show cells with different degrees of DNA damage

Table 1: Genotoxic (MN assay) assay of compound 6d

Sl. No.	Total no. of cells counted	% MN
Control	1000	**2.43**
VC	1000	**3.40**
10μg/ml	1000	**6.30**
25μg/ml	1000	**9.33**
50μg/ml	1022	**9.57**
100μg/ml	1015	**10.18**
200μg/ml	1022	**6.12**

3.5 In vitro DNA binding study of fluoro compound 6d

3.5.1. UV–Vis absorption studies

The absorption spectra of a constant concentration of DNA in the absence and presence of different concentrations of the compound **6d** were recorded. The DNA solution has an absorption peak at 259 nm [58]. From **Figure D** (please see Supporting Information), it can be observed that with increasing amounts of the compound **6d** to the DNA solution, the absorption intensity of this band increases (hyperchromism) without any apparent change in the position of band. The increase in the absorption intensity at i.e., λ_{max} = 259 nm may be due to purine and pyrimidine bases of DNA which were exposed because of the binding of the compound **6d** to DNA. This indicates that there is an interaction between compound **6d** and DNA which resulted in a slight change in the conformation of DNA.

3.6 Fluorescence study

3.6.1. Fluorescence quenching studies of compound 6d with BSA

Fluorescence spectroscopy is one of the constructive methods which can be used for the study of quenching mechanism, mode and strength of the interaction of BSA with the targeted compounds [59]. The BSA solution has a strong fluorescence emission peak at 344 nm when excited at 280 nm, which excitation wavelength of 280 nm would excite both tyrosine and tryptophan residues in proteins as discussed previously [60]. **Figure E** (please see Supporting Information) shows the fluorescence emission spectra of BSA in the absence and presence of the compound **6d** at room temperature. The fluorescence intensity (I) decreases regularly with increasing concentration of the compound **6d**, without any shift in the position of the maximum emission wavelength. The results indicate that the compound **6d** quenches the intrinsic fluorescence of BSA.

4 CONCLUSIONS

In summary, 2-(3-(aryl/heteroaryl)-1-phenyl-1*H*-pyrazol-4-yl)-2,3-dihydro-3 phenylquinazolin-4(1*H*)-ones derivatives **6a–d** was achieved by microwave irradiation. The key features of this method are benign reaction condition, short reaction time and excellent yield. Molecular docking study revealed that the designed molecular scaffold showed outstanding interaction with enzyme (PDB ID: 3V03). Significant cytotoxic and genotoxic effect was shown by compound **6d** on Ehrlich Ascites Carcinoma as concentration was increased. In UV-Visible studies, the interaction between compound **6d** and DNA resulted in a slight change in the conformation of DNA was observed. In Fluorescence studies the results show that the compound **6d** quenches the intrinsic fluorescence of BSA.

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