Pro-Apoptotic Activity of Novel 4-Anilinoquinazoline Derivatives Mediated by Up-regulation of Bax and Activation of Poly(ADP-ribose) Phosphatase in Ehrlich Ascites Carcinoma Cells

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Abstract

Quinazolines are very important class of heterocyclic compounds with antitumor properties. In search of novel anti-tumour agents, a series of 4-anilinoquinazolines were synthesized and characterized using proton and carbon-13 nuclear magnetic resonance, Fourier transform infrared and mass spectroscopic techniques. These compounds were evaluated for their cytotoxic effect on Ehrlich Ascites Carcinoma cells using 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide against Ehrlich ascites carcinoma cell lines. Among the tested compounds, compound N-3-(6,7-dimethoxyquinazoline-4-yl)amino)-phenyl)-4-nitrobenzene sulphonamide compound exhibited more potent activity with an IC50 value of $10.29 \pm 1.14 \mu M$ against EAC cell line. In vivo studies using compound N-3-(6,7-dimethoxyquinazoline-4-yl)amino)-phenyl)-4-nitrobenzene sulphonamide showed that there was a reduction in the body weight, ascites volume and decrease in cell number. Mice treated with compound N-3-(6,7-dimethoxyquinazoline-4-yl)amino)-phenyl)-4-nitrobenzene sulphonamide showed higher survivability compared with that of control treated mice. The cells treated with compound N-3-(6,7-dimethoxyquinazoline-4-yl)amino)-phenyl)-4-nitrobenzene sulphonamide also exhibited typical morphological changes of apoptotic damages. Further, compound N-3-(6,7-dimethoxyquinazoline-4-yl)amino)-phenyl)-4-nitrobenzene sulphonamide induced tumour cell death by activating proapoptotic protein Bax which activates caspase-3 and activated caspase –3 cleaves poly (ADP-ribose) polymerase causes DNA fragmentation. Thus, our results strongly conclude that our compound 4G acts as an anticancer agent by inducing apoptosis in Ehrlich ascites carcinoma cells.

Keywords: Quinazoline; Ehrlish ascites carcinoma cells; BAX; PARP; Apoptosis

Abbreviation: 4G: N-(3-((6,7-dimethoxyquinazoline-4-yl)amino)phenyl)-4-nitrobenzene sulphonamide.

Introduction

Apoptosis is referred as a programmed cell death and the most broadly studied mode of cell death [1]. It plays a crucial role in normal development and tissue homeostasis by facilitating the removal of unwanted, damaged or infected cells [1,2]. Impairment of the apoptotic signal promotes aberrant cell proliferation, agglomeration of genetic defects, ultimately resulting in tumorigenesis [3,4]. Therefore, induction of apoptosis in cancer cells is a target for developing potent anti-neoplastic drug in cancer treatment. It is an energy dependent process in which the characteristic morphological changes occur, it includes plasma membrane blebbing, exposure of phosphatidylserine at the external surface of the cell membrane, cell shrinkage, chromatin condensation and DNA fragmentation [3,4].

At the molecular level, apoptosis is tightly regulated by the activation of the Aspartate-specific cysteine protease (caspase) cascade. Activation of caspase cascade can be initiated through activation of the apoptotic pathway [5]. The distinguished extrinsic and intrinsic apoptotic pathway is based on the involvement of adaptor molecules and initiator caspases. These two pathways are interlinked and the molecules in one pathway influence the other pathway [6]. The mitochondrial pathway is regulated by activating pro-apoptotic proteins Bax and inhibiting the anti-apoptotic proteins Bcl of the Bcl-2 family [7-9]. Cellular stress induces pro-apoptotic protein Bax to migrate to the surface of the mitochondria, where it proceates pores in the mitochondrial membrane and permit the release of cytochrome C into cytoplasm. Once Cytochrome C is released, it activates initiator caspases-9 and results in the cleavage of effector pro-caspase -3, -6, and -7 [7,8,10]. The active caspase-3 and -7 specifically cleaves poly (ADP-ribose) polymerase (PARP) that will lead to the nucleosomal DNA fragmentation [11,12]. Cleavage of a definite number of key proteins is very important for the development of apoptotic events.

Quinazolines are big family of heterocyclic compounds, which have shown broad variety of biological activity profiles, such as analgesic [13], diuretic, antihypertensive [14], antimarialar [15], antibiotic, antitumoral [16-18], antiangiogenic [19] and many others. It is observed that the biological activity strongly depends on the type and the place of the substituents in their molecules. Even though various substitutions can be made on quinazoline ring, 4-amino substituted quinazolines are used as anti-cancer, anti-fungal, anti-inflammatory, anti-hypertensive.

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and anti-microbial agents [20-22]. Few of the 4-anilinoquinazolines have found to be potential and highly selective inhibitors of human immunoglobulin and epidermal growth factor receptor tyrosine kinase. Although the first signal transduction kinase inhibitor to proceed to clinical deployment was the pyrimidine derivative imatinib, many of the subsequent investigations have employed quinazoline derivatives, with 4-anilino derivatives being particularly dominant in this regard. Quinazolines are reported as inhibitors of EGFR auto phosphorylation with 4-anilino derivatives being particularly dominant in this regard.

Materials and Methods

Experimental methodology and characterization data of synthesized quinazoline derivatives 4(A-J)

The key intermediate compound 6,7-dimethoxy-quinazoline-4(3H)-one 1 compound was synthesised using the earlier reported procedure [24].

Synthesis of 6,7-dimethoxy-4-chloro quinazoline 2

To a stirred solution of compound 1 (10 mmol) in thionyl chloride (3 mL), DMF was added (2-3 drops) slowly. The reaction mixture was heated to reflux and stirred for about 2 h. Excess thionyl chloride was distilled off and the reaction mixture was quenched in ice with efficient stirring. The precipitate was filtered, washed with ice-water and dissolved in chloroform and filtered to remove insoluble impurities. Organic layer was concentrated under vacuum to obtain compound 2.

Synthesis of N-(3-aminophenyl)-6,7-dimethoxy-quinazolin-4-amine 3

To a stirred solution of 2 (10 mmol) in isopropyl alcohol (25 mL) meta-phenylenediamine was added (10 mmol). The reaction mass was heated to 60°C and stirred for about 3-4 h. Reaction was monitored by TLC. The reaction mass was quenched in ice water and heated to 60°C and stirred for about 3-4 h. Reaction was monitored by TLC. After completion, the reaction mass was quenched in ice water and heated to 60°C and stirred for about 3-4 h. Reaction was monitored by TLC. The reaction mass was quenched in ice water and heated to 60°C and stirred for about 3-4 h. Reaction was monitored by TLC. After completion, the reaction mass was quenched in ice water and heated to 60°C and stirred for about 3-4 h. Reaction was monitored by TLC. The precipitate was filtered, washed with ice-water and dissolved in chloroform and filtered to remove insoluble impurities. Organic layer was concentrated under vacuum to obtain compound 3.

General procedure for the preparation of compounds 4(A-J)

To a stirred solution of compound 3 (1 mmol) and triethylamine (1 mmol) in dichloromethane (10 mL), aryl sulfonyl chlorides (1 mmol) in dichloromethane (10 mL) δ: 10.41 (s, 1H, -NH), 9.62 (s, 1H, -NH), 8.53 (s, 1H, Ar-H), 8.22 (s, 1H, Ar-H), 7.94 (s, 1H, Ar-H), 7.80 (m, 1H, Ar-H), 7.61 (m, 2H, Ar-H), 7.55 (m, 2H, Ar-H), 7.47 (m, 2H, Ar-H), 7.20 (m, 1H, Ar-H), 3.97 (s, 3H, -OCH3). 13C NMR (DMSO-d6, 100 MHz) δ: 168.4, 164.0, 158.6, 154.5, 151.3, 146.6, 143.2, 137.5, 135.3, 130.5, 115.6, 112.9, 109.4, 108.2, 107.8, 102.7, 99.4, 56.2, 56.0. MS (ESI + ion): m/z = 454.8 Anal. Calcld for C15H12N3O4: C 58.14, H 4.21, N 12.33. Found: C 58.20, H 4.28, N 12.39. IR (KBr, cm-1): 3430, 3219, 1595

4-chloro-(6-(6,7-dimethoxyquinazoline-4-yl)amino)phenyl)-benzenesulfonamide 4D

White solid. M.p. 174-176°C. IR (KBr, cm-1): 3428, 3215, 1588, 1528. 1H NMR (DMSO-d6, 400 MHz) δ: 10.40 (s, 1H, -NH), 9.58 (s, 1H, -NH), 8.51 (s, 1H, Ar-H), 8.25 (s, 1H, Ar-H), 7.97 (s, 1H, Ar-H), 7.82 (m, 1H, Ar-H), 7.61 (m, 2H, Ar-H), 7.55 (d, J=8.0 Hz, 2H, Ar-H), 7.47 (d, J=8.0 Hz, 2H, Ar-H), 7.20 (m, 1H, Ar-H), 3.97 (s, 3H, -OCH3), 3.95 (s, 3H, -OCH3). 13C NMR (DMSO-d6, 100 MHz) δ: 168.4, 158.5, 154.6, 151.3, 146.6, 143.2, 141.1, 137.5, 133.4, 132.7, 130.5, 130.3, 129.5, 128.0, 119.2, 109.4, 108.2, 107.8, 102.7, 99.4, 56.2, 56.0. MS (ESI + ion): m/z = 506.0 Anal. Calcld for C28H23ClN4O6: C 51.28, H 3.83, N 11.44. IR (KBr, cm-1): 3430, 3215, 1595

4-(tert-butyl)-N-(3-(6,7-dimethoxyquinazoline-4-yl)amino)phenyl)benzenesulphonamide 4E

White solid. M.p. 154-156°C. IR (KBr, cm-1): 3432, 3219, 1595, 1289. 1H NMR (DMSO-d6, 400 MHz) δ: 10.41 (s, 1H, -NH), 9.56 (s, 1H, -NH), 8.49 (s, 1H, Ar-H), 8.22 (s, 1H, Ar-H), 7.95 (s, 1H, Ar-H), 7.80 (m, 1H, Ar-H), 7.61 (m, 2H, Ar-H), 7.52 (d, J=8.0 Hz, 2H, Ar-H), 7.44 (d, J=8.0 Hz, 2H, Ar-H), 7.18 (d, 1H, Ar-H), 3.97 (s, 3H, -OCH3), 3.95 (s, 3H, -OCH3), 1.32 (s, 9H, -(C(CH3)3). 13C NMR (DMSO-d6, 100 MHz) δ: 168.4, 158.5, 154.5, 151.3, 146.6, 143.2, 137.5, 137.3, 130.3, 129.0, 128.6, 112.8, 109.4, 108.2, 107.8, 102.7, 99.4, 56.2, 56.0. MS (ESI + ion): m/z = 478.0 Anal. Calcld for C28H23ClN4O6: C 51.11, H 4.07, N 11.90. Found: C 51.28, H 4.14, N 11.95.
N-(3-((6,7-dimethoxyquinazoline-4-yl)amino)phenyl)-2-nitrobenzenesulphonamide 4F

White solid. M.p. 182-184°C. IR (KBr, cm⁻¹): 3466, 3241, 1598, 1293. NMR (DMSO-d₆, 400 MHz) δ: 10.41 (s, 1H, -NH), 9.56 (s, 1H, -NH), 8.47 (s, 1H, Ar-H), 8.26 (s, 1H, Ar-H), 8.19 (s, 1H, Ar-H), 7.97 (s, 1H, Ar-H), 7.84 (d, J=8.0 Hz, 2H, Ar-H), 7.76 (s, 1H, Ar-H), 7.62 (m, 1H, Ar-H), 7.46 (m, 2H, Ar-H), 7.39 (m, 1H, Ar-H), 3.99 (s, 3H, -OCH₃), 3.96 (s, 3H, -OCH₃). ¹³C NMR (DMSO-d₆, 100 MHz) δ: 168.4, 158.5, 154.6, 151.2, 147.1, 146.5, 143.2, 137.5, 137.5, 131.7, 134.2, 132.8, 130.3, 128.2, 124.2, 112.9, 109.4, 108.2, 107.8, 102.7, 99.4, 56.2, 56.0. MS (ESI + ion): m/z = 348.8. Anal. Calc for C₂₇H₂₅N₄O₅S: C 54.89, H 4.04, N 14.55. Found: C 54.93, H 4.04, N 14.61.

N-(3-((6,7-dimethoxyquinazoline-4-yl)amino)phenyl)-3-nitrobenzenesulfoamide 4G

White solid. M.p. 190-192°C. IR (KBr, cm⁻¹): 3452, 3231, 1596, 1293. ¹H NMR (DMSO-d₆, 400 MHz) δ: 10.43 (s, 1H, -NH), 9.60 (s, 1H, -NH), 8.53 (s, 1H, Ar-H), 8.22 (s, 1H, Ar-H), 7.94 (s, 1H, Ar-H), 7.82 (m, 1H, Ar-H), 7.63 (d, J=8.0 Hz, 2H, Ar-H), 7.34 (m, 1H, Ar-H), 7.45 (d, J=8.0 Hz, 2H, Ar-H), 7.23 (m, 1H, Ar-H), 3.96 (s, 3H, -OCH₂). ¹³C NMR (DMSO-d₆, 100 MHz) δ: 168.5, 158.5, 151.4, 151.3, 146.5, 145.4, 143.2, 137.5, 130.3, 124.2, 112.9, 109.4, 108.2, 107.8, 102.7, 99.4, 56.2, 56.0. MS (ESI + ion): m/z = 481.7. Anal. Calc for C₂₇H₂₅N₄O₅S: C 54.88, H 3.98, N 14.55. Found: C 54.94, H 4.06, N 14.60.

N-(3-(6,7-dimethoxyquinazoline-4-yl)amino)phenyl)benzenesulphonamide 4H

White solid. M.p. 148-150°C. IR (KBr, cm⁻¹): 3436, 3241, 1602, 1282. ¹H NMR (DMSO-d₆, 400 MHz) δ: 10.33 (s, 1H, -NH), 9.57 (s, 1H, -NH), 8.46 (s, 1H, Ar-H), 8.23 (s, 1H, Ar-H), 8.11 (s, 1H, Ar-H), 7.93 (s, 1H, Ar-H), 7.81 (m, 2H, Ar-H), 7.77 (s, 1H, Ar-H), 7.56 (m, 1H, Ar-H), 7.43 (m, 2H, Ar-H), 7.49 (m, 1H, Ar-H), 3.97 (s, 3H, -OCH₃). ¹³C NMR (DMSO-d₆, 100 MHz) δ: 168.5, 158.5, 151.4, 146.5, 145.4, 143.2, 137.5, 130.3, 124.2, 112.9, 109.4, 108.2, 107.8, 102.7, 99.4, 56.2, 56.0. MS (ESI + ion): m/z = 481.7. Anal. Calc for C₂₇H₂₅N₄O₅S: C 54.88, H 3.98, N 14.55. Found: C 54.92, H 4.02, N 14.58.

N-(3-(6,7-dimethoxyquinazoline-4-yl)amino)phenyl)-2,3-stribenylbenzenesulphonamide 4I

White solid. M.p. 166-168°C. IR (KBr, cm⁻¹): 3466, 3239, 1608, 1277. ¹H NMR (DMSO-d₆, 400 MHz) δ: 10.36 (s, 1H, -NH), 9.57 (s, 1H, -NH), 8.46 (s, 1H, Ar-H), 8.27 (s, 1H, Ar-H), 7.96 (s, 1H, Ar-H), 7.64 (m, 2H, Ar-H), 7.59 (s, 1H, Ar-H), 7.50 (s, 1H, Ar-H), 7.45 (m, 1H, Ar-H), 7.21 (m, 1H, Ar-H), 2.38-2.42 (s, 9H, Ar-CH₃), 3.96 (s, 3H, -OCH₃).

Experimental Procedure for Biological Assays

Swiss albino mice were procured from the central animal facility, Department of studies in Zoology, University of Mysore, Mysore, India. EAC cells were obtained from NCCS (Pune).

Anti-proliferative assay

All the newly synthesized compounds (4(A-I)) were preliminarily evaluated for their anti-proliferative activity on EAC cell lines using MTT assay [25]. Ehrlich ascites carcinoma cell lines (Mouse mammary carcinoma) were cultured in Modified Eagle’s Medium (MEM), supplemented with 10% FBS at 37°C and humidified atmosphere containing 5% CO₂. Cells were seeded at a density of 1 × 10⁴ cells/well in a 96 well plate and allowed to attach for 24 hours. The media was removed and treated with different concentration (1-100 μg) of compound for 24 hours. Then, 10 μL of MTT solution (5 mg/ml) was added to each well and incubated at 37°C for 4 hours. Then, medium was removed, and 200 μL of dimethyl sulfoxide (DMSO) was added to each well in order to solubilize formazan crystals. The absorbance was measured at 570 nm by micro plate reader. The IC₅₀ value was defined as the concentration that caused 50% inhibition of cell proliferation.

In-vivo treatment of compound 4G

EAC (5 × 10⁴ cells/mouse) cells were injected intraperitoneally into six to eight weeks old swiss albino mice and the animals showed a significant increase in the body weight over the growth period. To determine, whether the compound inhibit tumor growth, 4G (100 mg/kg) was administrated in the EAC bearing mice on every alternate day starting from 6th day of inoculation. The growth of tumor was monitored by taking the body weight every day. The animals were sacrificed on 11th day. The volume of ascites from both treated and control were noted. The other group animals were used to study the survivability after treatment until their death [26].

Studies on cell morphology

Giems and nuclear staining were performed and visualized using light and fluorescent (Leitz-DIAPLAN) microscope. Both 4G treated and untreated cells were harvested from mice, fixed in methanol: acetic acid (3:1) and were smeared on glass slide and air-dried in humidified chamber. The cells were hydrated with PBS and stained with Giemsa or mixture of (1:1) acridine orange-ethidium bromide (4 µg/ml). These cells were washed with PBS and visualized under light or Leitz-DIAPLAN fluorescent microscope respectively [27-29].

Measurement of cell viability

In-vivo treated and untreated cells were harvested from mice and the packed cells were diluted (1:6 ratio). Cell viability was assessed by counting the number of viable cells using haemocytometer by 0.4% trypan blue dye exclusion method. cells that picked up the dye were considered to be dead [26].

Apoptosis assay by FACS

Phycoerythrin conjugated antibody against activated caspase-3 (CloneC92-605) was used to label caspase-3 according to the manufacturer’s instructions (BD Pharmingen); samples were analysed...
by flow cytometry. Briefly, the cells were harvested from 4G treated and control group, where in cisplatin was used as positive control. Then the cells were re-suspended in BD Cytofix/Cytoprot solution (BD Pharmingen) and incubated on ice for 20 minutes. The cells were washed with 1X BD Perm/Wash buffer (BD Pharmingen) and labelled with phycoerythrin- conjugated anti--activated caspase- 3 antibody at room temperature for 30 minutes before analysis by flow cytometry (Beckman Coulter Inc.) [30].

DNA fragmentation assay

This is a technique which is used to visualize the endonuclease cleavage of apoptosis [31,32]. This assay involves extraction of DNA from treated and untreated cells using phenol-chloroform method. Cells were lysed using 10% SDS, incubated for 30 min at 37°C. The cell lysate was precipitated using 8M potassium acetate at 4°C for 1 hr. the supernatant was extracted by phenol: chloroform: isooamyl alcohol (25:24:1). DNA was precipitated by adding 1.2 volume of ice-cold ethanol to the supernatant and was dissolved in TE buffer. The DNA was treated with RNase at 37°C for 1 hr. The extracted DNA was separated by 1.2% agarose gel electrophoresis and visualized by staining with ethidium bromide under UV trans illuminator and documented using UVP-BioDoc-ItTM system.

Caspase -3 inhibition assay

EAC cells harvested from control mice were pre-incubated with or without caspase-3 inhibitor AC-DEVE CHO (100 µM) at 37°C for 1 hr. Subsequently the cells were treated with 4G and incubated for another 2 hrs. at 37°C. DNA was isolated and the fragmentation was visualized by 1.5% agarose gel electrophoresis [30].

RNA isolation and reverse transcription-polymerase chain reaction

Total RNA from control and test sample was prepared using the Trizol reagent (Invitrogen) according to the manufacturer’s protocol and was used for RT-PCR using the Super-Script III One-Step RT-PCR system (Invitrogen) [33,34]. We used 1 µg of RNA for reverse transcription-polymerase chain reaction (RT-PCR) using the Super-Script III One-Step RT-PCR system (Invitrogen Life Technologies). Briefly, the complementary DNAs were synthesized at 50°C for 30 minutes followed by incubation at 94°C for 2 minutes. Subsequently, 30 cycles of PCR were carried out with denaturation at 94°C for 45 seconds, annealing at 53°C for 45 seconds, and extension at 72°C for 1.5 minute, followed by a final incubation at 72°C for 7 minutes. For amplifying Bax and Bcl, the following primers sets (Sigma) were used. Bax PCR primers set (Product No. B8304) Reverse primer sequence (3’ antisense): 5‘-CAT CTT CTT CCA GAT GGT GA-3’ Forward primer sequence (5’ sense): 5‘-GTT TCA TCC AGG AGG GAC GAG CAG-3’ and bcl-2 PCR primers set (Product No. B9179) Reverse primer sequence (3’ antisense): 5’-GAG ACA GCC AGG AGA AAT CA-3’ Forward primer sequence (5’ sense): 5‘-CCCT GTG GAT GAC GTA GTA GC-3’. GADPH (Product No. P7732) Reverse primer sequence (3’ antisense): 5’-GCC CTG CTG CAC CAG CTT C-3’ Forward primer sequence (5’ sense): 5’-TGC MTC CTG CAC CAC AAA CT-3’ where M = A or C Y = T or C that was used as a loading control was amplified using primers that have been previously published and it served as a loading control. The PCR products were then separated on a 1.5% agarose gel and results were documented.

Western blotting

EAC cells treated and untreated with 4G compounds were harvested and lysed for 1 h at 4°C in a lysis buffer (20 mM Tris pH 7.5, 2 mM EDTA, 3 mM EGTA, 2 mM dithiothreitol (DTT), 250 mM sucrose, 0.1 mM phenylmethylsulfonyl fluoride, 1% Triton X-100) containing a protease inhibitor cocktail. Protein (50 µg) was resolved on 12.5% SDS gel and transferred to PVDF membrane. Equal protein loading was controlled by Ponceau Red staining of membranes. For immunoblotting, anti-PARP (ab-72805) and cytochrome c (7H8): sc-13560 primary antibodies were used followed by the addition of HRP-conjugated specific secondary antibody. Actin (ab-3280) was used as internal loading control. The protein bands were developed and visualized by enhanced chemiluminescence [35,36].

Results and Discussion

Chemistry

The synthesis of Quinazoline derivatives 4(A-J) was carried out as per synthetic procedure represented in Scheme 1. The key intermediate compound 6,7-dimethoxy-quinazolin-4(3H)-one 1 was prepared using the earlier reported procedure. Compound 2 was treated with thiouyl chloride in presence few drops of DMF to get chloro derivative 2, which then coupled with 3-aminoaniline in isopropyl alcohol at 80°C to get meta-phenylene diamine derivative 3. Structure of the compound 3 was confirmed by the appearance of –NH character in the ¹H NMR spectrum of compound 3 around δ 9.5 and a peak at δ 10.5 corresponding to –NH. Compound 3 was then treated with different aryl sulfonyl chlorides in presence of triethylamine in dichloromethane to get crude products 4(A-J), which were further purified by using silica gel column. The structure of compounds 4(A-J) were confirmed by the disappearance of –NH peak in the ¹H NMR spectrum of compounds and appearance of –NH peak around δ 10.4. Structures and yields of the compounds are presented in Table 1.

4G induces cytotoxic effect in EAC cells

The synthesized compounds 4(A-J) were evaluated for their anti-proliferative activity on EAC cell lines using MTT assay. Cisplatin was used as the positive control. All the synthesized compounds significantly inhibited the proliferation of cancer cells in a dose-dependent manner (1-100 µM) after 24 hrs. of incubation. IC₅₀ values for the cytotoxic effects of various synthetic compounds 4(A-J) are shown in Table 1. The compounds 4A, 4B, 4C, 4E, 4F and 4H showed good activity with IC₅₀ values 27.4 ± 2.14, 18.3 ± 1.94, 24.2 ± 1.91, 22.5 ± 2.13,12.9 ± 2.1, 10.29 ± 1.14 and 14.8 ± 1.91 µM respectively, whereas the IC₅₀ value for cisplatin was 6.4 ± 1.45 µM. These values suggest that the activities of the synthesized compounds are very close to that of the

Scheme 1: Synthesis of compounds 4(A-J): i) Thiouyl chloride, DMF, reflux, 2 h; ii) meta-phenylenediamine, Isopropyl alcohol, reflux, 3-4 h; iii) Substituted benzene sulfonyl chlorides, triethyl amine, dichloromethane, 0°C to RT, 4-5 h.
| Compound | Structure | % Yield | IC_{50} value (µM) |
|----------|-----------|---------|-------------------|
| 4A       | ![Structure of 4A](image) | 59      | 27.4 ± 2.14       |
| 4B       | ![Structure of 4B](image) | 55      | 18.3 ± 1.94       |
| 4C       | ![Structure of 4C](image) | 60      | 24.2 ± 1.94       |
| 4D       | ![Structure of 4D](image) | 65      | NA                |
| 4E       | ![Structure of 4E](image) | 59      | 22.5 ± 2.13       |
| 4F       | ![Structure of 4F](image) | 65      | 12.9 ± 2.1        |
| 4G       | ![Structure of 4G](image) | 60      | 10.29 ± 1.14      |
| 4H       | ![Structure of 4H](image) | 52      | 14.8 ± 1.91       |
| 4I       | ![Structure of 4I](image) | 55      | NA                |
| 4J       | ![Structure of 4J](image) | 60      | NA                |

NA: Not Active; *Yield corresponds to the final step of synthesis

**Table 1:** Structure, % of yield and IC_{50} (µM ± SD) value of compounds 4(A-J).
standard cisplatin. Compounds 4A and 4C with halogen substituents on phenyl ring at various positions (2, 4 and 5) are least active (24.2 ± 1.94 to 27.4 ± 2.14 µM). Activating groups like methoxy, on para position of phenyl ring, which release electrons by resonance in 4B enhanced the activity to 18 ± 1.94 µM. Besides, inductively electron releasing 4-tert butyl group in 4E is less active (22.5 ± 2.13 µM). On the other hand, deactivating group like nitro, irrespective of its position on phenyl ring of compounds 4F-H, enhanced the activity (10.29 ± 1.14 to 14.8 ± 1.91 µM). Thus, compounds 4F-H showed 1-2 fold high IC₅₀ values compared to that of the standard cisplatin. In particular, the order of activity is 4-nitro (4G) > 2-nitro (4F) > 3-nitro (4H). Compound 4G, which showed the highest activity (10.29 ± 1.14 µM) was taken for further detailed studies.

**Induction of anti-tumor activity by 4G significantly increases survivability of treated mice**

We adopted EAC murine model to evaluate the anti-proliferative and pro-apoptotic activity of the compound 4G. To determine the In-vivo effect on cell growth, we investigated the effect of 4G compound on body weight, ascites volume and survivability in the treated and untreated tumor bearing mice. We observed there was a gradual decrease in the body weight (Figure 1A), ascites volume (Figure 1B) and there was a significant increase (53%) in life span of 4G treated mice in comparison with control (Figure 1C). Decrease in tumour growth also correlates with significant increase in life span. Our lead compound 4G inhibits the tumor growth and increases survivability of the mice (Figure 1D). The prolongation of lifespan of 4G treated EAC bearing mice is a very important and reliable criterion.

**4G induces apoptosis in EAC cells**

Morphological evaluation of Microscopic examination revealed that, the cells stained with Giemsa displayed cell shrinkage, formation of small blebs and apoptotic bodies in 4G treated cells in comparison with control (Figure 2A). Cytological analysis showed nuclear condensation when 4G treated cells are stained with acridine orange/ ethidium bromide, which is an important feature of apoptosis as compared to the untreated EAC cells (Figure 2B). Activation of proteolytic enzymes such as cysteine, serine proteases and proteinases may correlate with the main apoptotic events includes plasma membrane blebbing, shrinkage of the cytoplasm, dilation of endoplasmic reticulum, nuclear chromatin condensation and DNA fragmentation. The nuclear condensation and formation of apoptotic bodies indicates that the compound 4G induce apoptosis in treated cells when compared to that of control cells that showed intact nuclear architecture.

**4G inhibits cell viability and induces caspase-3 activation:**

Trypan blue dye exclusion method was used to determine the cell viability. The result shows a significant decrease in viability 4G treated cells when compared to untreated cells (Figure 3A). Activation of caspase-3 pathway is a hallmark of apoptosis and can be used in cellular assays to quantify activators and inhibitors of the “death cascade.” The response is both time and concentration dependent, suggesting that multiple pathways play a role in triggering the caspase-3 activation. To evaluate the effect of 4G in treated cells, a significant proportion of cells were induced to undergo apoptosis through caspase-3 activation and the effects were determined by the flow cytometric analysis. FACS analysis revealed about 45% cells showed active caspase-3 where as in control 2.7%. Cisplatin was used as positive control shows that 63% of activated caspase-3 (Figure 3B).

**4G induced apoptosis is dependent on caspase-3 activation**

The appraisal of apoptotic pathway was further carried out by determining the DNA ladder patterns. EAC cells treated with 4G showed characteristics of DNA laddering that had higher apoptotic cells while the DNA of control cells exhibited minimum fragmentation. Cisplatin was used as a positive control (Figure 4A). The downstream signaling pathway revealed that the activation of caspase-3 in 4G treatment induced EAC cell death. In order to check the involvement of caspase-3 activator of endonuclease, the cells were treated with or without caspase-3-inhibitor (Ac-DEVD-CHO), prior to the treatment with 4G compounds [30]. The results clearly suggest that, the DNA fragmentation is due to increased endonuclease activity but the Ac-DEVD-CHO, a specific inhibitor of caspase-3 enzyme inhibited the DNA fragmentation (Figure 4B). DNA degradation and caspase-3 activation...
inhibition assays showed that the 4G induce caspase-3 mediated apoptosis in EAC cells.

**BAX activation by 4G leads to cleavage of PARP in EAC cells**

The signaling pathway by which compound 4G induces apoptosis in EAC cells was depicted by assessing the changes in expression levels of apoptotic proteins. Bcl-2 superfamily plays an important role in apoptosis; it acts as activator or inhibitor. Of this, the anti-apoptotic protein Bcl-2 and pro-apoptotic protein BAX play important role in cell death. In mitochondrial pathway compound 4G induced apoptosis was investigated, by examining the level of Bcl-2 and BAX expression, which are the crucial regulators of the apoptotic pathway using RT-PCR. In the present study, compound 4G increased mRNA expression level of BAX and reduced level of Bcl-2 was observed (Figures 5A and 5B). So, our data suggest that the compound 4G may disturb the Bcl-2 /BAX ratio and leads to apoptosis of EAC cells. Activation of caspase was determined by the cleavage of specific caspase substrates. Activation of caspase-3 results in the cleavage of key proteins such as poly-ADP-ribose polymerase (PARP), lamins and inhibitor of caspase-activated DNase in downstream cascade, resulting in programmed cell death. The nuclear enzyme PARP is cleaved by executioner caspase-3 and -7 [35,36]. The western blot data clearly revealed the cleavage of PARP protein in treated cells where as in control cells that was intact (Figure 5C). Cisplatin treated cells served as a positive control and were found to have effectively down regulated the expression of Bcl-2, BAX and PARP cleavage. This provides a strong indication that compound induces apoptosis in EAC cells.

**Conclusion**

In conclusion, the synthesized novel quinazoline derivatives have shown anticancer potential against EAC cells. Compound 4G possess the capability of inducing mitochondrial pathway in EAC cells, which is well regulated by caspase enzymes. Moreover, the active role of mitochondrial dependent pathway has been studied and was further confirmed by increasing BAX pro-apoptotic protein level, activation of caspase-3 and cleavage of PARP protein by caspase-3 proteins. Our findings strongly suggest that the anti-cancer and pro-apoptotic potential of compound 4G can be used as lead for developing therapeutic agent for treating cancer.

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