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Elucidation of Structure-activity Relationship of 2-Quinolone Derivatives and Exploration of Their Antitumor Potential Through Bax-induced Apoptotic Pathway

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3-Aryl-2-quinolone derivates were extensively investigated for their inhibition of farnesyl transferase. Taking this as a cue, we studied the other possible mechanism of antitumor activity of 2-quinolone derivates. A series of new 2-quinolone derivatives have been synthesized and screened for their cytotoxicity by trypan blue assay on Ehrlich ascites carcinoma cells and MTT assay on MCF-7 cells. Compound 1a (nJST) was found to be more effective in both studies with the lowest CTC_{50} value among all nine synthesized compounds. This compound was further screened on four different cell lines, viz. human breast adenocarcinoma (MCF-7, MDA-MB-231), colon cancer (HCT-15), murine melanoma (B16F10) cell lines for 24 and 48 h. The CTC_{50} value of the compound was found to be <10 μM. Compound 1a induced DNA damage which was revealed by DNA fragmentation studies and further confirmed by nuclear staining. The compound also showed significant elevation in Bax and reduction Bcl-2 gene expression levels. Acute toxicity study in mice indicated that the compound is safe till 2000 mg/kg. Two different doses 50 and 100 mg/kg were selected and studied in Ehrlich ascites carcinoma model of cancer and have shown significant improvement in survival time and hematological parameters.

Key words: 2-quinolone, cytotoxicity, DNA fragmentation, Ehrlich ascites carcinoma, MTT assay

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Treatment for cancer is still being a challenge for medical world. Many researches are going on to get a safe and effective treatment for this disease. 2-Quinolones are one of the molecules in this category. Joseph *et al.* initiated the exploration of anticancer potential of this moiety by reporting a series of 2-quinolones with 3-aryl and *N*-alkyl substitutions of which 12 compounds were exhibiting cytotoxicity (CTC_{50}) of more than 10 μM on MCF-7 (human breast cancer) cell line. However, these compounds were found to be nontoxic in *in vivo* toxicity determination and effective in *in vivo* model of MXT mouse mammary adenocarcinoma (1).

The leading molecule of this category, tipifarnib, is still in clinical trial stage. This molecule is also have 3-aryl and *N*-methylation substitutions and exhibits cytotoxicity in breast cancer cell lines MDA-MB-231 and BT-474 with a CTC_{50} value <30 μM (2). Moreover, it is active orally and causes apoptosis in myeloid leukemia cell line (3).

Apoptosis plays a central role in study of carcinogenesis and drug development for the cancer therapy. It is a regulated evolutionary conserved programme of cell suicide. Disturbance in this physiological programme prolongs the life of cell and leads to carcinogenesis. In cancer cells, the apoptosis diminishes and causes dominance of anti-apoptotic protein. Mitochondrial-mediated apoptosis is controlled by anti-apoptotic (Bcl-2) and proapoptotic (Bax and Bad) proteins of Bcl-2 family. Overexpression of Bcl-2 occurs in 40–80% of human breast cancers (4).

These studies added rising interest in developing and evaluating anticancer activity of 2-quinolone derivatives through apoptotic pathway. Among quinolones, most of the anticancer compounds have a 3-aryl substitution. However, to our knowledge nobody has explored the anticancer activity of 3-methyl substituted quinolones. Previously, we synthesized and evaluated compounds **1b–i** and identified them as promising antimicrobial and antioxidant agents (5). But none of the compounds was tested for their cytotoxic potential against cancer cell lines. With this rationale and our continuing effort to explore the biological activity of 2-quinolones, we report here the synthesis and pharmacological evaluation of the compounds **1a–i** containing in their structures a 2-quinolone moiety as the pharmacophore and a functionally diverse side chain through amide linkage as the lipophilicity contributor to the scaffold (Figure 1). Present study was also

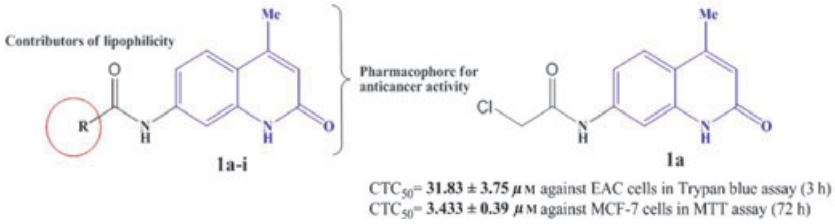


Figure 1: Structures of the compounds.

aimed to establish a relationship between structures and their cytotoxic potential, finally exploring the mechanism of action of the most active compound.

Methods and Materials

Materials

Chloro acetyl chloride, minimum essential medium (MEM), and MTT(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) were obtained from Sigma Chemicals Co., St. Louis, MO, USA and DMSO from S.D. Fine Chemicals, Mumbai, India. Other chemicals of analytical grade were obtained from RFCL Limited (Rankem), New Delhi, India, and S.D. Fine Chemicals, Mumbai, India.

Test drugs preparation

All *in vitro* cytotoxic studies were performed using DMSO (0.1%) as solvent. In control samples, an equimolar amount of DMSO was used to rule out its effect on cytotoxicity. For *in vivo* screening, the test drugs were suspended in 0.25% carboxymethylcellulose, freshly prepared before use, and given orally. The standard anticancer drug (Cisplatin) was also injected intraperitoneally.

Cell line and culture media

Breast cancer cell line (MCF-7, MDA-MB-231), murine melanoma cell line B16F10, and colon cancer cell line (HCT-15) used in the study were procured from National Centre for Cell Science, Pune, India, and maintained by regular sub-culturing. Cells were routinely grown in 25 cm^2 culture flasks (Techno Plastic Products, Trasadingen, Switzerland) with loosened caps containing MEM supplemented with 10% fetal calf serum (FCS) and 50 μg/mL gentamicin sulfate at 37 °C in an atmosphere of humidified air containing 5% CO_2 in a CO_2 incubator.

The Ehrlich ascites carcinoma (EAC) cells were obtained from Dr Ramdasan Kutan (Director, Amala Cancer Research Center, Amala Nagar, Thrissur, Kerala, India). They were maintained and propagated by serial intraperitoneal transplantation in an aseptic environment. Cells propagated for 12–14 days were used in the experiment.

Animals

Eight- to 10-week-old Swiss albino mice weighing 20–30 g selected from an inbred colony maintained in the Central Animal Research

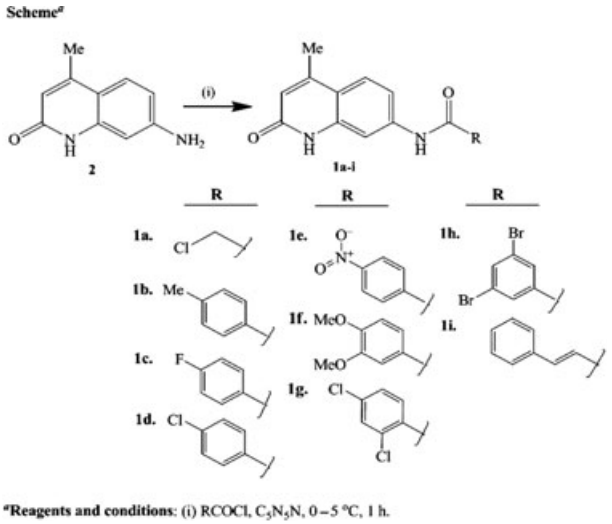


Figure 2: Synthesis of compounds **1a**–**i** (Scheme).

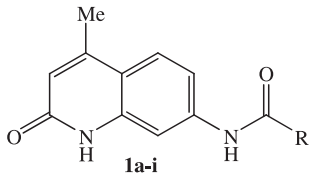
Facility of Manipal University were employed for the study. The mice were kept in polypropylene cages (4 per cage) in an air-conditioned room maintained at a comfortable temperature (23 ± 2 °C) with a 12-h light-dark cycle. The experiments on mice were approved by the Institutional Animal Ethical Committee (IAEC) (No. IAEC-KMC/06/2006-2007) and were conducted according to the guidelines of CPCSEA.

Test compounds

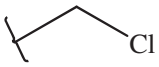
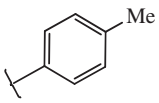
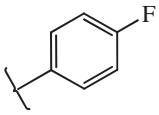
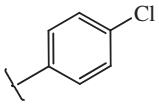
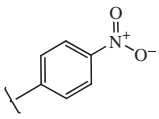
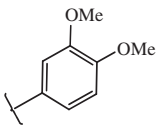
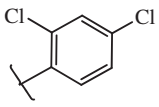
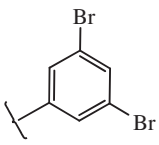
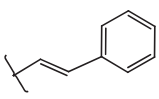
The compounds **1a**–**i** illustrated in scheme (Figure 2) were synthesized and characterized in our laboratory (Table 1). Among them, compounds **1b**–**i** are already reported in our previous publication(2). However, compound **1a** (nJST) is claimed to be novel and its details are described below.

Preparation of 2-chloro-N-(4-methyl-2-oxo-1,2-dihydroquinolin-7-yl) acetamide {Compound **1a**}: To a solution of 7-amino 4-methyl 2-quinolone (0.4 g, 2.29 mmol) in pyridine (4 mL), chloroacetyl chloride (0.51 g, 4.59 mmol, 0.368 mL) was added in drop-wise manner at 0–5 °C and kept for stirring at 5–10 °C. Completion of the reaction (1 h) was confirmed by TLC using acetone/chloroform (70:30) as the mobile phase. Then, reaction mixture was poured in cold water (15 mL) and extracted with ethyl acetate (3 × 15 mL). The organic layers were pooled, washed with dilute HCl (5 mL), brine (10 mL), dried over anhydrous sodium sulfate, and evaporated under

Table 1: Synthesis of compounds **1a–i**



1a-i

Compound code	R	% Yield	MP (°C)	R_f value[a]
1a		25	>310	0.70
1b		70	320	0.73
1c		68	315	0.70
1d		85	>320	0.72
1e		85	>320	0.72
1f		74	318	0.62
1g		88	318	0.79
1h		83	>310	0.77
1i		66	>320	0.71

[a]Solvent system – acetone/chloroform, 70:30.

vacuum to afford 0.36-g crude oil. Crude compound was purified by column chromatography using silica 100–200 and pet. ether/ethyl acetate (6:4) as the mobile phase to afford 0.144 g off white solid, Yield: 25%; Melting Point: > 310 °C; R_f: 0.7 (acetone/chloroform, 70:30). Λmax (methanol): 254 nm. IR (KBr, per cm): 3216 (N-H str), 3018 (Ar-H str), 2968 (-CH_3 str), 2852 (-CH_2 str), 1676 (-CO str of open amide), 1641 (-CO str of cyclic amide), 1614 (C=C str), 1543, 1454, 1396 (Ar-C=C str). Mass Spectra: 250 $[M]^+$, 252 $[M]^{++}$, 174 $[M^+\text{-}C_2H_2ClO]^+$, 201 $[M^+\text{-}C_3H_2ClO_2]^+$, 103 $[M^+\text{-}C_6H_6ClO_2N]^+$. ^{1}H NMR (300 MHz, DMSO-d_6): δ 11.6 (s, 1H, NH), 10.6 (s, 1H, NH), 7.75 (d, J = 3 Hz, 1H), 7.66 (d, J = 9 Hz, 1H), 7.36–7.32 (dd, J = 9 Hz and 3Hz, 1H), 6.28 (s, 1H), 4.3 (s, 2H), 2.4 (s, 3H).

Experimental pharmacology

In vitro cytotoxic activities (Trypan blue dye exclusion method)

All the synthesized compounds were screened for *in vitro* cytotoxicity in EAC cells at 3 h of drug incubation. The EAC cells were maintained and propagated intraperitoneally by serial transplantation in adult male Swiss albino mice. Ascitic fluid was withdrawn from EAC inoculated mice without getting contaminated with blood. Cells were washed with phosphate buffer saline, and stock was adjusted to 1×10^6cells/mL. In a final volume of 1 mL, the cells were incubated with the desired drug concentration for 3 h at 37 °C. After the incubation period, 0.1 mL of trypan blue was added in the incubation mixture and mixed well. Total numbers of dead and live cells were counted using hemocytometer. The percentage cytotoxicity was calculated according to the standard method (6).

In vitro cytotoxic activities (MTT assay)

Exponentially growing cells were plated in 96-well plates (10^4 cells/well in 100 μL of medium) and incubated for 24 h for attachment. Desired concentrations of test compounds were prepared in 0.1% DMSO prior to the experiment. The entire reactant mixture was diluted with media, and cells were exposed to different concentrations of drugs (1, 10, 100, and 200 μM). After 72 h, media were removed and cell cultures were incubated with 100 μL MTT reagent (1 mg/mL) for 4 h at 37 °C, and the formazan produced by the viable cells was solubilized by addition of 100 μL DMSO. The suspension was placed on micro-vibrator for 5 min and absorbance was recorded at 540 nm by the microplate reader and percentage cytotoxicity was calculated (7). Same method had been used for compound **1a** for (1, 5, 10, 25, 50, and 100 μM) on four different cell line viz., MCF-7, B16F10, MDA-MB-231, and HCT-15 for 24 h and 48 h.

DNA fragmentation study

DNA fragmentation study of compound **1a** was performed by exposing different concentration of the compound (2 and 4 μM) on MCF-7 cell line. DNA was extracted from the treated and untreated samples using phenol chloroform extraction. Then, DNA fragmentation pattern was studied with the help of agarose gel electrophoresis (8).

Nuclear staining

0.1 million cells were seeded in each well of 24-well plates with minimal essential medium containing 10% FBS. After 24 h, cells were treated with **1a** at 2 and 4 μM for 24 h. The plates were incubated at 37 °C in 5% CO_2 atmosphere. After overnight incubation, medium from wells was discarded and cells were washed with PBS. The cells were fixed with 1 mL of methanol (90%) at −20 °C for 20 min. The methanol was removed and air dried. Fixed cells were washed with ice cold phosphate buffer saline (pH 7.4) three times. The cells were incubated with PBS containing 1% BSA and 0.1% triton X-100 at 37 °C for 30 min. Plate was washed with PBS three times and 400 μL of acridine orange (0.01% in PBS pH-7.4) was added and incubated at 37 °C for 20 min. The plate was washed thrice with PBS and observed under the fluorescent microscope for any nuclear morphological changes (9).

Isolation of total RNA and reverse transcription polymerase chain reaction (RT-PCR)

MCF-7 cells were grown on 25 cm^2 bottles at 1×10^6 cells/mL concentrations for 24 h at 37 °C in a humidified atmosphere of 5% CO_2. The cells were then exposed to two different concentrations of **1a** (2 and 4 μM) or the medium alone (as normal). After 24 h of drug treatment, total RNA was extracted from these cells using trizol reagent and quantified by DNA protein enzyme analyser (Shimadzu, Japan). cDNA synthesis and amplification was performed by PCR apparatus (Eppendorf Germany) in a volume of 50 μL comprising of 2 μL total RNA, 2 μL oligo(dT), 25 μL of RT-PCR master mix, 3 μL of 50 mM $Mn(OAc)_2$, 2 μL of respective forward and reverse primers, and 16 μL of RNAse free water. Polymerase activation was carried out at 90 °C for 30 seconds and reverse transcription was performed at 60 °C for 30 min. *Thermus thermophilus* DNA polymerase enzyme was used for cDNA synthesis step and PCR amplification step. The sequences of the PCR primers for Bax were 5′-CCA AGA AGC TGA GCG AGT GTC TC-3′ (forward) and 5′-AGT TGC CAT CAG CAA ACA TGT CA-3′ (reverse), Bcl-2 were 5′-GGA GCG TCA ACA GGG AGA TG-3′ (forward) and 5′-GAT GCC GGT TCA GGT ACT CAG-3′ (reverse), and the sequences for GAPDH (NM_017008) were 5′-CCA AGA AGC TGA GCG AGT GTC TC-3′ (forward) and 5′-CCT GCT TCA CCA CCT TCT TG -3′ (reverse). The cycle condition of PCR amplification process consisted of 40 cycles, including denaturation at 94 °C for 1 min, annealing at 60 °C for GAPDH, 51 °C for Bax and Bcl-2, 46 °C for p53 for 30 seconds, and extension at 72 °C for 1 min with one cycle of final extension at 60 °C for 7 min. The predicted sizes of the amplified products of Bax, Bcl-2, and GAPDH were 487, 127, and 349 bp, respectively. Equal amounts of corresponding products of Bax, Bcl-2, and GAPDH were separated by 1.5% agarose gel electrophoresis (Genei, Bangalore, India) and optical densities of ethidium bromide-stained DNA bands were quantified by Alpha Innotech software, USA (10).

Toxicological study (OECD 425 Guidelines)

Compound **1a** that has shown promising activity in all the studies was subjected to toxicological studies as per OECD 425 guidelines to obtain safe dose in mice.

In vivo cytotoxic activity in Swiss albino mice (EAC cells)

A known number of viable EAC cells (2.5×10^6cells/mice) were injected intraperitoneally into each animal in an aseptic condition and the day of tumor inoculation was considered as day 0 (11). All the experiments of tumor-bearing mice were conducted 24 h after EAC transplantation, and that day was considered as day 1. Twenty-four hours after EAC tumor cell inoculation, the tumor-bearing animals were randomly divided into desired groups and treated with test compound or vehicle. The test compound was administered intraperitoneally on day 1st, 3rd, 5th, 7th, 9th, 11th, and 13th of tumor inoculation. Cisplatin (single dose of 3.5 mg/kg, i.p.) was injected on day 1st which served as standard and following parameters were assessed.

Percentage increase in weight as compared to day '0' weight (12). Upon weighing the animals on the day of inoculation and after once in 3 days in the post inoculation period, the % increase in weight was calculated as follows.

$$\%\text{increase in weight} = [(\text{Animal weight on respective day} / \text{animal weight on day 0}) - 1] \times 100$$

Mean survival time (MST) and percentage increase in mean life span (% IMLS). Total number of days an animal survived from the day of tumor inoculation was counted and MST was calculated (13). Subsequently, % IMLS was calculated as follow,

$$[(\text{Mean survival time of treated group/mean survival time of control group}) - 1] \times 100$$

Hematological studies. Whole blood count was assessed on day 15th. Blood was withdrawn from orbital plexus and used to estimate the WBC, RBC, and hemoglobin count following the standard procedures (14).

Statistical analysis

Data represent the mean ± SEM of the indicated number of experiments. Statistical analysis and graphs of the data were prepared by one-way ANOVA (GraphPad Prism Version 5.02, Instat Software, La Jolla, CA, USA) followed by Tukey's *post hoc* test. A value of $p < 0.05$, $p < 0.01$, $p < 0.001$ was considered to be significant.

Results

Short-term in vitro cytotoxicity in EAC cells by Trypan blue exclusion assay

The % cytotoxicity of tumor cells was assessed at 3 h of drug incubation in EAC cells. Considerable cytotoxicity was observed in compounds **1a, 1b**, **1d**, **1e**, **1g**, and **1i** (Table 2).

Table 2: Short-term *in vitro* cytotoxicity in EAC cells by Trypan blue exclusion assay and *in vitro* cytotoxicity in MCF-7 cells by MTT assay

Compound code	CTC50 (μM) in Trypan blue assay[a]	CTC50 (μM) in MTT assay[b]	Predicted CLogP[c]
1a	31.83 ± 3.75	3.433 ± 0.39	1.288
1b	106.7 ± 4.17	110.2 ± 4.48	2.663
1c	>1000	1.289 ± 2.10	2.377
1d	98.91 ± 6.19	314.42 ± 11.03	2.947
1e	146.64 ± 3.15	110.7 ± 5.95	2.114
1f	>1000	>1000	1.935
1g	63.81 ± 4.28	50.79 ± 2.63	2.855
1h	>1000	6.360	3.98
1i	178.8 ± 4.36	305.3	3.178

EAC, Ehrlich ascites carcinoma.
Average of five determinations, four replicates.
[a]On EAC cell after 3 h of incubation;
[b]In MCF-7 cell line after 72 h of incubation;
[c]ChemDraw-2008.

In vitro *cytotoxicity by MTT assay*

To check the cytotoxicity in human cancer cell, the breast adenocarcinoma (MCF-7) cell line was selected. Five compounds (**1a**, **1b**, **1c**, **1e** and **1g**) were found effective after 72 h of incubation. However, only compound **1a** could inhibit the cell growth at <10 μM (Table 2). This compound was studied further on four different cell lines, viz. MCF-7, MDA-MB-231, B16F10, and HCT-15 cell lines. It was found to be active at 24 and 48 h. The CTC_{50} value was <10 μM in all the four cell lines (Table 3).

DNA fragmentation study

The cell number in compound **1a**-treated cells (4 μM) was found to be decreased compared to control, which suggests its role in causing DNA damage leading to cell death. This was confirmed by DNA damage. In normal untreated group, single clear band was observed, that is, DNA was intact. Increasing drug concentration caused DNA damage, which is clearly visible in lane 2 and 3 which may be attributed to the damage caused by compound **1a** (2 and 4 μM) (Figure 3).

Table 3: *In vitro* cytotoxicity of compound **1a** by MTT assay against four different cell lines

	CTC_{50} (μM) of compound **1a**	
Cell line	24 h	48 h
MDAMB	2.15 ± 0.36	1.47 ± 0.24
HCT	2.13 ± 0.36	1.6 ± 0.23
MCF-7	3.62 ± 0.37	4.43 ± 0.26
B16F10	1.46 ± 0.28	1.21 ± 0.34

Average of five determinations, four replicates.

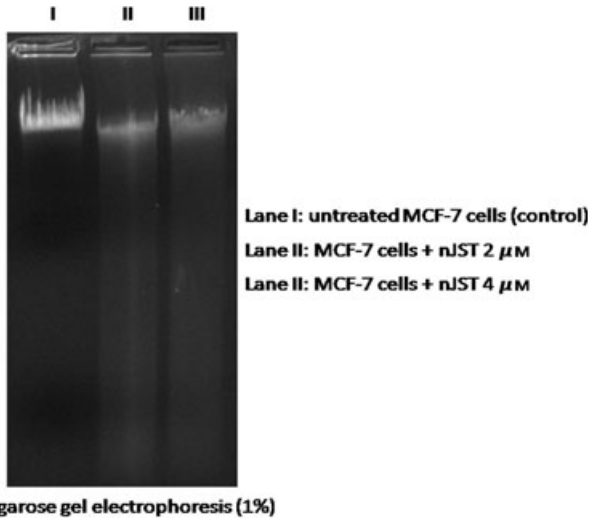


Figure 3: DNA fragmentation.

Nuclear staining

Alteration in the nuclear structure of the drug-treated cells is one of the important aspects to study nuclear morphology. To visualize nuclear morphology, nuclear staining was carried out using dye acridine orange. The study was carried out against MCF-7 cells. Nucleus in control cells was very much intact, round, or oval in shape, without any condensation and blabbing. Cytoplasm was normal with intact cell membrane and cytoplasm disintegration was not observed. When cells were treated with compound **1a** (2 and 4 μM), they showed typical apoptotic morphological changes such as nuclear and cytoplasmic condensation, loss of cell volume, and nuclear fragmentation (Figure 4).

Reverse transcriptase PCR analysis

Reverse transcriptase PCR was used to analyze the levels of Bax and Bcl-2 mRNA levels in MCF-7 cells treated with compound **1a** (2 and 4 μM). Amplification of Bax and Bcl-2 was carried out with help of specific primers using cDNA as a template strand prepared from MCF-7 cells, and the specificity was confirmed using agarose gel electrophoresis.

Bax and Bcl-2 mRNA expression

The mRNA levels of Bax and Bcl-2 from MCF-7 cells are shown in Figure 5. The levels of Bax in MCF-7 cells with compound **1a** treatment increased significantly compared with untreated normal cells ($p < 0.001$). Compound **1a** at 4 μM concentration caused a significant decrease of Bcl-2 mRNA levels when compared with that of the normal cells ($p < 0.001$).

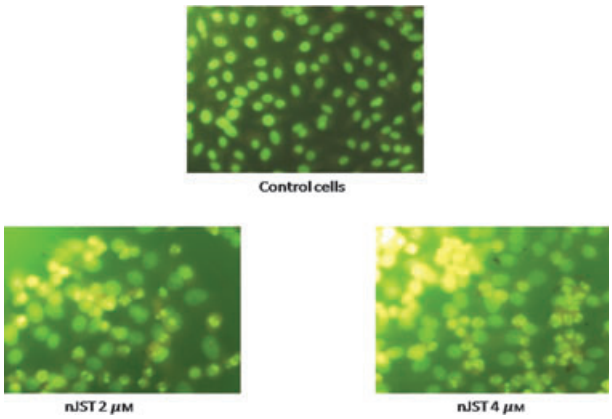


Figure 4: Nuclear Staining.

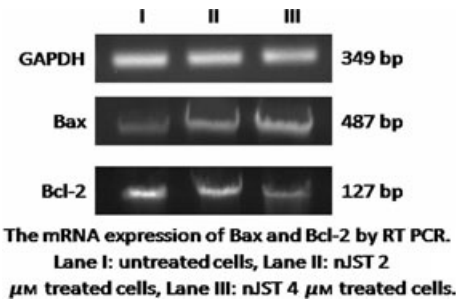


Figure 5: Bax and Bcl-2 expression.

Change in body weight in EAC inoculated mice

A significant gain in body weight was observed in EAC inoculated control mice, while maximum gain (47%) was observed within 15 days. Cisplatin administration on day 1st significantly ($p < 0.05$) reduced the elevated body weight. Drug treatment (compound **1a** at 50 mg/kg and 100 mg/kg) was monitored every alternate day for body weight. In the treated mice, no significant change in body weight was observed till 5th day. All the treatment showed a significant reduction in bodyweight compared to control from day 9 onwards. On day 7, only cisplatin treatment and **1a** treatment at 100 mg/kg have shown the significant reduction in body weight compared to sham control ($p < 0.05$) (Figure 6).

Effect on survival time in EAC inoculated mice

In EAC inoculated control mice, MST was found to be 16.16, while median survival time was 16. In cisplatin treatment, a significant increase in MST (38.16) and % IMLS (138.1) was observed. **1a** has significantly increased MST to 24.16 days ($p < 0.05$) and 27.5 days ($p < 0.01$), while % IMLS was 48.5 and 70.1 days for 50 mg/kg and 100 mg/kg, respectively. Median survival time for cisplatin, compound **1a** at 50 and 100 mg/kg was found to be 40, 24, and 27 day, respectively (Figures 7 and 8).

Effect of synthesized compounds on hematological parameters in EAC inoculated mice

WBC increased about twofold in control EAC inoculated mice, which was significantly ($p < 0.001$) reversed by cisplatin treatment and **1a** treatment at 100 mg/kg ($p < 0.01$). Tumor induction in control mice reduced the RBC count compared to normal animals ($p < 0.01$), which was significantly improved by tested compound

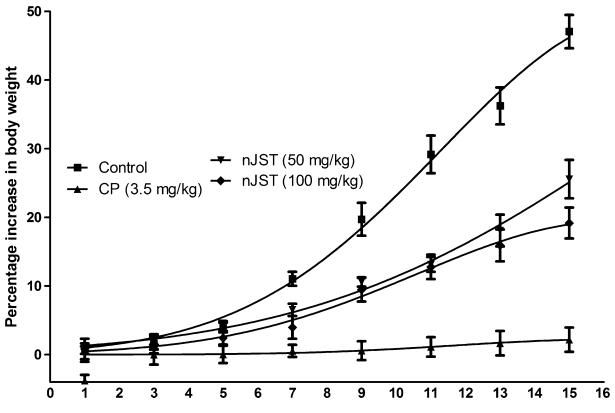


Figure 6: Percentage increase in body weight. All the values are mean ± SEM of six animals.

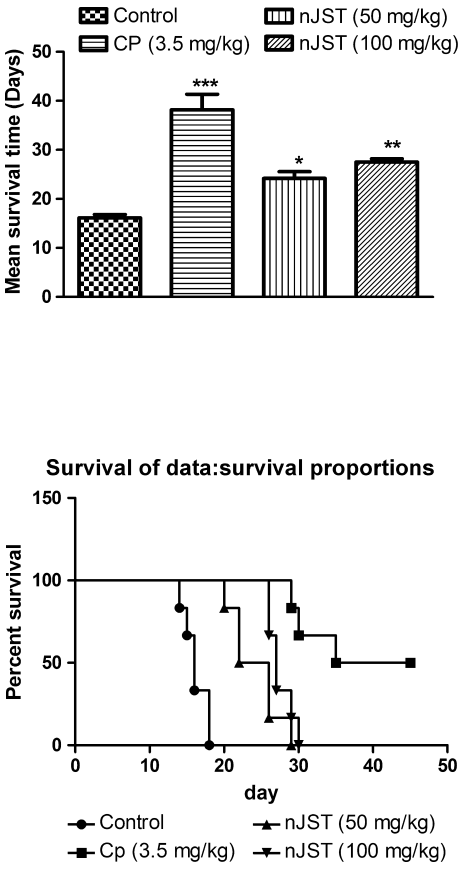


Figure 8: Percentage survival. The data represent six animals in each group.

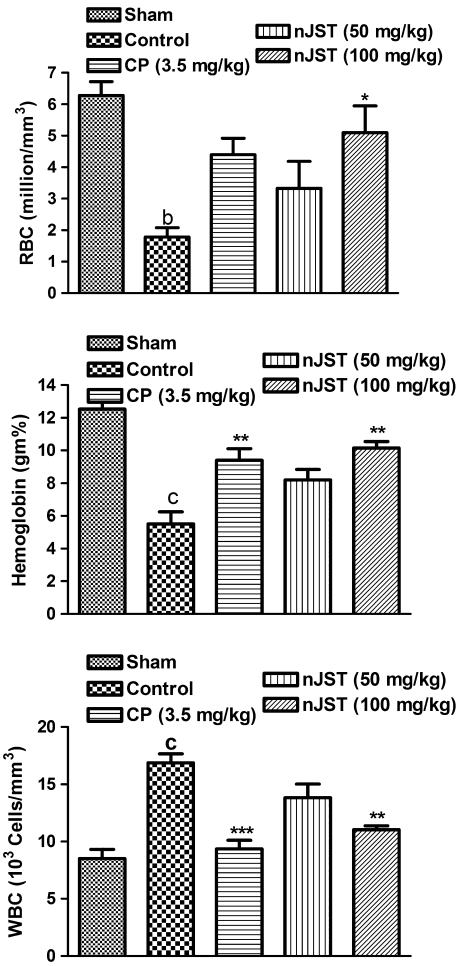


Figure 9: Hematological parameters. All the values are mean ± SEM of three animals, where *p < 0.05, **p < 0.01, ***p < 0.001 are compared to control and [a]p < 0.05, [b]p < 0.01, [c]p < 0.001 are compared to Sham animals.

at 100 mg/kg (p < 0.05). Tumor development in the animals caused significant anemia (decrease in hemoglobin content and RBC count), which was significantly (p < 0.01) reversed by cisplatin treatment and compound **1a** (100 mg/kg) (p < 0.01) (Figure 9).

Discussion

Chemistry

The solution phase syntheses of compounds **1a**–**i** are depicted in scheme (Figure 2). Previously reported by Jayshree *et al.*, 2010, compounds **1b**–**i** were resynthesized and confirmed by comparing with the reported R_f values and melting points. Novel analog of the series compound **1a** was synthesized by acetylating 7-amino-4-methylquinolin-2(1H)-one (compound **2**) using the same protocol applied for the preparation of compounds **1b**–**i** and characterized by 1HNMR, GC-MS, IR, and UV.

Pharmacology

Anticancer drug development has become an extremely competitive and expensive process with high rate of failures. So, the present study was designed to explore the possible *in vitro* and *in vivo* anticancer activity of already existing quinolone compounds, which have previously been screened for other activity (5). Generally, anticancer activity of most of the chemotherapeutic agents is owing to their cytotoxicity (15). Trypan blue exclusion assay and MTT assay are the widely used method for evaluation of cytotoxicity.

Therefore, a series of nine novel substituted *N*-(4-methyl-2-oxo-1,2 dihydroquinolin-7-yl) amides were synthesized and subjected to Trypan blue assay against EAC cells and MTT assay against MCF-7 cell line. The CTC_{50} was determined after 3 h of incubation in Trypan blue assay and after 72 h in MTT assay. CTC_{50} of our compounds are summarized in Table 2. Of the nine compounds synthesized, compound **1a** was found to be most active with CTC_{50} of 31.83 μM against EAC cells and compound **1c** was the most potent (CTC_{50} = 1.289 μM) to inhibit the proliferation of MCF-5 cell lines. Compounds with electron-withdrawing group substituted at the phenyl ring appear to be more activity against MCF-7 cell line. However, functional modifications on the phenyl ring show a complex relationship with the anticancer activity against EAC cells in Trypan blue assay.

Structure-activity relationships

To explore the structure-activity relationships (SAR) of substituted *N*-(4-methyl-2-oxo-1,2 dihydroquinolin-7-yl) amides, functional modifications have been carried out at the amino group.

The compound **1a** with haloalkyl chain attached to the parent nucleus through an amide linkage at amino group was considered as a reasonable starting point for SAR studies. It showed significant anticancer activity of CTC_{50} = 31.83 μM against EAC cells in Trypan blue assay and CTC_{50} = 3.433 μM against MCF-7 cell line in MTT assay. Encouraged with this result, a more lipophilic (ClogP = 2.663) '4-toluyl' substituent was introduced in place of haloalkyl substituent of compound **1a** to afford compound **1b**. To our surprise, this modification drastically lowered the anticancer activity in multifold against both the cancer cells. Because the electron-donating substituent on phenyl ring was inactive, analogs of **1b** in which the 4-methyl substituent on phenyl ring was altered with electron-withdrawing group like 4-fluorine (compound **1c**) and 4-chlorine (compound **1d**) to investigate the cytotoxicity. Remarkably, compound **1c** exhibited an excellent improvement in CTC_{50} of 1.289 μM against MCF-7 cell line where as compound **1d** showed a little improvement in CTC_{50} of 98.91 μM against the proliferation of EAC cells. The promising results of compound **1c** and **1d** prompted us to probe the effect of a charged $-NO_2$ group in place of halogens on the phenyl ring. Nevertheless, it was failed to improve the anticancer activity. Introduction of dimethoxy functionality in the phenyl ring of our moiety also resulted in equally frustrating activity (CTC_{50} □ 1000 μM against both the cells). Discouraged with the insensitivity of electron-donating functionality on anticancer activity, strong electron-withdrawing functional groups like –Cl and –Br were placed twice in the phenyl to afford compounds **1g** and **1h**, respectively. Interestingly, with this simple modification, compound **1h** showed a CTC50 of 6.360 μM against MCF-7 cell lines, whereas compound **1g** was moderately active against both the cancer cells. However, introduction of a spacer in between amide functionality and the phenyl ring of our moiety obliterated the anticancer activity. On the contrary, our SAR study reveals that nature and position of functional groups on the phenyl ring influence the anticancer activity of *N*-(4-methyl-2-oxo-1,2 dihydroquinolin-7-yl) amides. In addition, we speculate that the functional group that increases the acidity of our parent nucleus is essential for the anticancer activity. However, in our SAR study, we could not establish a strong correlation between anticancer activity and Log P of the compounds.

As compound **1a** has shown better cytotoxicity than other compounds (CTC_{50} = 3.433 ± 0.39 μM), it has been screened further on four cell lines including MCF-7 for 24 and 48 h. It has also shown promising result on the remaining cell lines at both time intervals with CTC_{50} <10 μM. This compound has been further studied for DNA fragmentation assay on MCF-7 cell line. It has shown a band with laddering, which indicates its action responsible for DNA damage. Nuclear staining experiments were performed using acridine orange staining dye. Compound **1a** at 2 and 4 μM concentration was tested against MCF-7 cells, and nuclear staining images showed induction of apoptosis such as membrane blebbing, cytoplasmic condensation, and nuclear fragmentation. Further reverse transcriptase PCR (RT-PCR) was performed to confirm the involvement of apoptosis in MCF-7 cells. RNA was isolated from MCF-7 cells with and without treatment with **1a** 2 and 4 μM. Primers specific for Bax and Bcl-2 were used to confirm the involvement of apoptosis. RT-PCR results showed upregulation of Bax expression and downregulation of Bcl-2 expression when compared with that of untreated MCF-7 cells. It has been reported that there is a strong relationship in apoptosis and farnesyl transferase inhibition (16–18). Hence, this finding supports that molecule **1a** may be a farnesyl transferase inhibitor.

Ehrlich tumor is a hurriedly growing carcinoma with very aggressive behavior (19). It can grow in almost all strains of mice. The tumor implantation induces a local inflammatory reaction, with increasing vascular permeability, resulting in an intense edema formation, cellular migration, and progressive ascitic fluid formation (20). The ascitic fluid is essential for tumor growth, as it constitutes a direct nutritional source for tumor cells. Drug treatment was given on 1st, 3rd, 5th, 7th, 9th, 11th, and 13th day of inoculation and checked for anticancer activity at these time points. Compound **1a** has been found to be significantly effective in reducing the body weight and also improved MST significantly compared to control EAC injected mice. Prolongation in the life span of tumor inoculated mice is a reliable criterion for judging the anticancer activity of any drug (13). In untreated mice, EAC inoculation causes 100% mortality within 20 days, which is supported by our present data. An enhancement of life span by 25% or more over that of control was considered as effective antitumor response (13). Treatment with compound **1a** at 50 and 100 mg/kg has delayed the onset of mortality and increased the life span of EAC inoculated mice by 49.48% and 70.1%, respectively.

Significant decrease in hemoglobin and RBC (owing to hypoxic condition) with parallel increase in WBC [may be owing to the influence of tumor cell (antigen) on the immune system] has been observed in EAC inoculated control mice. Compound **1a** at 100 mg/kg significantly reduced the elevated WBC count and increased RBC number compared to EAC inoculated control mice. The reversal of WBC indicates protective action on the hemopoietic system and supports the antitumor activity of the compound. Usually, in cancer chemotherapy, the major problems that are encountered include myelosuppression and anemia (21). The results had clearly shown that compound **1a** at 100 mg/kg was found to be significantly active in improving the hemoglobin content and RBC count to normal levels.

Conclusion

The study shows that 2-quinolone without 3-aryl substitution can also be a potential moiety for anticancer research. Upregulation of Bax expression and downregulation of BCl-2 expression in cancer cells is a very important event in apoptosis. Hence, we propose Bax-induced apoptosis as a potential alternative mechanism of 2-quinolone derivatives other than farnesyl transferase inhibition. Further, they have the significant cytotoxic potential and are safe as indicated by acute toxicity activity. Compound **1a** merits further investigation to explore in molecular mechanisms.

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