Anticancer Potential of 3-(Arylideneamino)-2-Phenylquinazoline-4(3H)-One Derivatives

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Key Words
Quinazolinone derivatives • Apoptosis • Anti-tumor

Abstract
Different quinazoline derivatives have showed wide spectrum of pharmacological activities. Some 3-(arylideneamino)-phenylquinazoline-4(3H)-ones have been reported to possess antimicrobial activity. The present study has been undertaken to evaluate the anticancer effect of these quinazolinone derivatives. The quinazolinone derivatives were synthesized as reported earlier. Compounds containing NO₂, OH, OCH₃, or OH and OCH₃ as substituent(s) on the arylideneamino group were named as P(3a), P(3b), P(3c), and P(3d) respectively. Out of these, P(3a) and P(3d) showed better cytotoxic activity than P(3b) and P(3c) on a panel of six cancer cell lines of different origin, namely, B16F10, MiaPaCa-2, HCT116, HeLa, MCF7, and HepG2, though the effect was higher in B16F10, HCT116, and MCF7 cells. P(3a) and P(3d) induced death of B16F10 and HCT116 cells was associated with characteristic apoptotic changes like cell shrinkage, nuclear condensation, DNA fragmentation, and annexin V binding. Also, cell cycle arrest at G1 phase, alteration of caspase-3, caspase-9, Bcl-2 and PARP levels, loss of mitochondrial membrane potential, and enhanced level of cytosolic cytochrome c were observed in treated B16F10 cells. Treatment with multiple doses of P(3a) significantly increased the survival rate of B16F10 tumor bearing BALB/c mice by suppressing the volume of tumor while decreasing microvascular density and mitotic index of the tumor cells.

Introduction
Cancer is alarmingly increasing in the developing world [1]. Unregulated cell growth without apoptosis leads to cancer. Treatment options for cancer patients have evolved with the advent of novel biological agents capable of inducing apoptosis. Apoptosis is a highly complex cascade of cellular events that are characterized by chromatin condensation, DNA fragmentation, phosphatidylserine exposure, cytoplasmic membrane blebbing, and cell shrinkage [2-4].
Caspases, members of cysteine protease family, are the principal biochemical effectors of apoptotic pathways (intrinsic and extrinsic) that are activated by cleavage. The final phase of apoptosis is characterized by activation of executioner caspases (e.g. caspase-3), shared by both of these pathways [5, 6]. The anti-apoptotic protein Bcl-2 and the proapoptotic protein Bax are important modulators in regulating cell death [7].

Quinazoline and its derivatives are a class of heterocyclic compounds of importance in medicine and agriculture because of their antibacterial, antifungal, anti-HIV [8, 9], antihelminthic [10], CNS depressant [11], antitubercular [12], anti-inflammatory, diuretic, anti-convulsant, antiallergic, antihypertensive and antiparkinsonian [13-16] properties. Many of the derivatives act as anticancer agents by interacting with the cytoskeleton, inhibiting DNA topoisomerase, and thus inducing apoptosis [17-19]. Recently four aniloquinazoline derivatives have been reported to show apoptosis inducing and antiproliferative activity on leukemic cells [20]. NTCHMTQ, a new series of quinazoline derivatives, showed cytotoxic activity in human cervical cancer cells [21]. 2, 4, 6-Trisubstituted quinazoline derivatives showed cytotoxic activity against some leukemic and melanoma cell lines [22] and few 2,4-dibenzyl aminooquinazolines have been found to induce selective apoptosis on some human cells [23]. Some 3-(arylidenamino)-2-phenylquinazoline-4(3H)-one derivatives have been reported to show antibacterial activity [24]. We have synthesized four derivatives by condensation of nitro benzaldehyde, vanillin, anisaldehyde or salicylaldehyde with 3-amino-2-phenylquinazolin-4(3H)-ones, which were named as P(3a), P(3b), P(3c) and P(3d) respectively. The substituents present in the phenyl ring are shown in Table 1. The synthetic route is summarized in scheme 1.

**Preparation of 3-(arylidenamino)-2-phenylquinazoline-4(3H)-ones**

3-(Arylidenamino)-2-phenylquinazoline-4(3H)-one derivatives were synthesized as described earlier [24]. Briefly, anthranilic acid was treated with benzoyl chloride in the presence of pyridine to undergo cyclization forming 2-phenyl-4H-benzo[d][1,3]oxazin-4-one, which on condensation with hydrazine hydrate yielded 3-amino-2-phenylquinazolin-4(3H)-one. This was then treated with different substituted benzaldehydes like nitrobenzaldehyde, vanillin, anisaldehyde, and salicylaldehyde separately in ethanol to form the corresponding 3-(arylidenamino)-2-phenylquinazolin-4(3H)-ones, which were harvested with 0.025% trypsin and 0.52 mM EDTA in phosphate buffered saline (PBS), and seeded at desired density to allow them to re-equilibrate a day before the start of experiment.

**Assessment of cell morphology**

Cells (3 × 10⁴/well) grown in 6-well plates in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic (PSN) at 37°C in a humidified atmosphere under 5% CO₂. After 75-80% confluency, cells were plated in 96 well plates and treated with or without derivatives. Morphological changes were observed with an inverted phase contrast microscope (Model: OLYMPUS IX 70, Olympus Optical Co. Ltd., Sibuya-ku, Tokyo, Japan) and images were acquired.

**Materials and Methods**

**Chemicals**

Chemicals used are: Anthranilic acid (Sigma Aldrich India), pyridine, anisaldehyde, salicylaldehyde, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), p-nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate system (NBT-BCIP) (SRL, India). Benzoyl chloride, sodium bicarbonate, hydrazine hydrate, 4-nitrobenzaldehyde, and vanillin (Merck, India). Cell lines were obtained from NCCS Pune, India. Dulbecco’s Modified Eagle Medium (DMEM), fetal bovine serum (FBS), penicillin streptomycin neomycin (PSN) antibiotic, trypsin and ethylenediaminetetraacetic acid (EDTA) were obtained from Gibco BRL (Grand Island, NY, USA). Tissue culture plastic wares were obtained from NUNC (Roskilde, Denmark) and Bradford protein assay reagent from Fermentus, EU. DAPI (4’,6-diamidino-2-phenylindole dihydrochloride), acridine orange (AO), and ethidium bromide (EtBr) were procured from Invitrogen, California. Antibodies were obtained from Santa Cruz Biotechnology, Inc., USA and eBioscience Inc., San Diego, USA. The caspase-3 and caspase-9 activity assay kit, propidium iodide (PI), and caspase inhibitors Z-DEVD and Z-LEDH were purchased from Sigma Aldrich MO, USA.

**Cell culture**

B16F10 (murine melanoma), MCF-7 (Human breast adenocarcinoma), HCT 116 (Human colorectal carcinoma), HepG2 (Human hepatocellular carcinoma), MiaPaCa-2 (Human pancreatic carcinoma), and HeLa (Human cervical cancer) cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic (PSN) at 37°C in a humidified atmosphere under 5% CO₂. After 75-80% confluency, cells were seeded at desired density to allow them to re-equilibrate a day before the start of experiment.

**MTT assay and trypan blue exclusion test**

MTT assay was done to evaluate cell viability [25]. The cells were plated in 96 well plates and treated with or without different concentrations of derivatives for 24 h. Four hours after the addition of MTT, cells were lysed and formazan was solubilized with acidic isopropanol and the absorbance of the solution was measured at 595 nm using an ELISA reader. Briefly, after treatment with P(3a) and P(3d) for 24 h, B16F10, HCT 116 and MCF-7 cells were stained with 0.4% trypan blue and approximately 100 cells were counted at various fields in haemocytometer for each experiment.
Fluorescence microscopy

For the detection of nuclear damage or chromatin condensation, treated and untreated cells were stained with 10 μg/ml of DAPI [26]. Cells were stained with AO/EtBr in order to distinguish the live, apoptotic and necrotic ones, and observed under fluorescence microscope; images were then acquired with excitation and emission wavelengths of 488 and 550 nm respectively [27].

Detection of cell death by ELISA

Cell death was assayed using an ELISA based cell detection kit from Roche Molecular Biochemicals (Mannheim, Germany).

Quantification of apoptosis using Annexin-V

Apoptosis was assayed by using an Annexin-V FITC apoptosis detection kit (Calbiochem, CA, USA). Briefly, cells were treated with or without derivatives, then washed and stained with PI and Annexin-V-FITC in accordance with the manufacturer’s instructions. The percentages of live, apoptotic and necrotic cells were determined by flow cytometric method (Beckton Dickinson, San Jose, CA, USA). Data from 10⁶ cells were analyzed for each sample.

Cell cycle analysis

B16F10 cells were seeded in 6-well plates and treated with the derivatives. Harvested cells were fixed over-night in 70% ethanol at 4°C, collected by centrifugation, resuspended in phosphate-buffered saline (PBS) containing 25 μg/ml RNase and 0.5% Triton-X100, and incubated for 1 h at 37°C. Cells were stained with 50 μg/ml PI for 15 min at 4°C and analysed by flow cytometer (10⁴ cells were counted).

Caspase-3, caspase -9 and cytochrome c assay

Levels of procaspases and cytosolic cytochrome c were assayed by commercially available colorimetric assay kits (Bio Vision Research Products, Mountain View, CA) in accordance with the manufacturer’s instructions.

Western blot analysis

The cell lysates were separated by 10% SDS-PAGE, then transferred to PVDF membranes (Millipore, Bedford, MA) using standard electroblotting procedures. Membranes were then blocked and immunolabeled overnight at 4°C with primary antibodies. Alkaline phosphatase conjugated secondary antibodies and NBT-BCIP were used as chromogenic substrates [28].

Mitochondrial membrane potential measurement

To measure the mitochondrial membrane potential, treated or untreated cells were washed and incubated with Rhodamine 123 (5 μg ml⁻¹). Emission at 535 nm was measured in a spectrofluorometer (LS50B, Perkin Elmer).

In vivo antitumor activity of derivatives

B16F10 cells were injected subcutaneously (s.c.) on the right flank of BALB/c mice for tumor induction. Care and maintenance of animals were done in adherence to the guidelines of the Institutional Animal Care and Use Committee. In toxicity study, intraperitoneal (i.p.) injection of 5, 50, 100 and 200 mg/kg/dose of P(3a) did not show any abnormalities. The animal were divided into three groups (n, where n = 6). One group of tumor bearing mice were given i.p. injections (15 or 30 mg/kg/day) of P(3a) as per Wilcoxon method [29]. Tumor volume was measured with caliper as a function of time, and the survival rate was noted [30].

Statistical analysis

All values are expressed as mean ± SD. Statistical significance was compared between various treatment groups and controls using the one-way analysis of variance (ANOVA). Data were considered statistically significant when P values were <0.01.

Results

Effect of arylideneamino derivatives on the growth of cancer cell lines

Treatment of P(3a), P(3b), P(3c), and P(3d) ranging concentration from 0 to 100 μM on a panel of five human cancer cell lines of different origins, namely, MCF-7, HCT116, HepG2, MiaPaCa-2, HeLa, and one murine melanoma cell line, B16F10 for 24 h showed various degree of cell death as shown in Fig.1A (a-f). IC₅₀ values of the compounds on 24 h on these cell lines were shown in the Table 2. P(3a) and P(3d) showed higher toxicity on...
B16F10, and HCT116 cells (IC_{50} values are within 25 µM. Also, IC_{50} value of P(3d) on MCF-7 is low (21.55±4.4). Cytotoxicity of P(3a) and P(3d) on rest of the cell lines and that of P(3b) and P(3c) on all the cell lines tested here found to be relatively (IC_{50} values are greater than 25 µM). Percentage of viable cells counted from trypan blue exclusion test in P(3a) and P(3d) treated B16F10, MCF-7, HCT116 cells as seen in Table 3 matches with the cell viability study done from MTT assay. The data are represented as mean ± SD from triplicate independent experiments. B. Morphological and nuclear changes in (a) B16F10 and (b) HCT 116 cells after 30 µM of P(3a) or P(3d) treatment for 24 h. Upper row: Morphological changes seen under light microscope. Middle and lower row: Nuclear changes seen under fluorescence microscopy after DAPI and A.O./EtBr staining respectively.

B16F10, and HCT116 cells (IC_{50} values are within 25 µM. Also, IC_{50} value of P(3d) on MCF-7 is low (21.55±4.4). Cytotoxicity of P(3a) and P(3d) on rest of the cell lines and that of P(3b) and P(3c) on all the cell lines tested here found to be relatively (IC_{50} values are greater than 25 µM). Percentage of viable cells counted from trypan blue exclusion test in P(3a) and P(3d) treated B16F10, MCF-7, HCT116 cells as seen in Table 3 matches with the cell viability study done from MTT assay. Optimum cell death on B16F10, MCF-7, HCT116 cells was within 75-90% in 24 h with 60 µM of each of P(3a) or P(3d). 30 µM of each of P(3a) or P(3d) showed optimum percent (75-90%) of cell death on the two highly susceptible cell lines B16F10, and HCT 116 of in 36 h (data not shown). Vehicle control, i.e. 0.2% DMSO, did not show any cytotoxic effect on the cell lines tested here. Cytotoxicity was not detected with 100 µM of P(3a) or P(3d) in normal blood cells. Thus, the two new arylideneamino derivatives, P(3a) and P(3d) may be potent cytotoxic agents for some selected cancer cells.

B16F10, and HCT 116 cells were treated with P(3a) or P(3d) and examined for the mechanism of cell death. Apoptosis is desirable for cancer cell death. So, the involvement of apoptosis in P(3a) and P(3d) induced death of B16F10, and HCT 116 was tested. Treatment with 30 µM P(3a) and P(3d) for 24 h showed characteristic apoptotic changes like cell rounding and cell shrinkage under light microscope. Fluorescence microscopy study
using nuclear staining dye DAPI and AO/EtBr showed the presence of condensed and fragmented nuclei unlike untreated control. DAPI staining showed bluish intact nuclei in control and bright fragmented nuclei in the treated cells. AO/EtBr treatment showed green intact nuclei in control cells, but greenish yellow, yellowish red and reddish fragmented nuclei in P(3a) and P(3d) treated cells (Fig. 1B, a-b). Thus the microscopic study indicated that P(3a) and P(3d) induced death of B16F10 and HCT 116 cells may involve apoptosis.

**P(3a) and P(3d) enhance annexin V positive cells with cell cycle arrest at G0/G1 phase in B16F10 cells**

At initial phase of apoptosis, phosphatidyl serine is exposed from inner membrane to outer membrane. Phosphatidyl serine exposure in the outer membrane was measured by Flow cytometric analysis using annexin V-FITC in B16F10 cells. The percentage of viable cells was significantly lowered and the total number of apoptotic cells (in LR & UR) remarkably increased compared to the control following treatment of the cells with 30 µM of P(3a) or P(3d) for 24 h and 36 h. This study confirms that P(3a) and P(3d) induce apoptosis in B16F10 cells (Fig. 2A).

Cell cycle consisting of four regulations phases (G0/G1, S, G2, and M), is an integrated part of cell growth. Apoptosis is followed by cell cycle arrest at any one of these phases. So, status of cell cycle arrest in P(3a) and P(3d) treated B16F10 cells was monitored by Flow cytometric analysis using PI. Treatment of 30 µM of P(3a) or P(3d) for 24 h and 36 h showed higher number of cells at G0/G1 phase. This study indicated that the active derivatives P(3a) and P(3d) induced apoptosis of

| Cell Lines | IC_{50} P(3a) | IC_{50} P(3b) | IC_{50} P(3c) | IC_{50} P(3d) |
|------------|---------------|---------------|---------------|---------------|
| B16F10     | 23.17±6.1     | 44.53±4.1     | 42.27±4.1     | 23.28±1.1     |
| HeLa       | 39.76±5.1     | 31.93±5.1     | 32.84±5.2     | 32.27±4.1     |
| HCT        | 19.27±4.1     | 71.12±3.1     | 57.04±4.9     | 20.63±2.1     |
| MCF-7      | 36.84±7.1     | 39.44±6.1     | 43.75±3.8     | 21.55±4.4     |
| HepG2      | 34.25±5.1     | 45.37±3.1     | 32.42±2.1     | 36.41±5.1     |
| Mia-Pa-Ca  | 35.1±3.1      | 29.49±5.1     | 30.55±6.1     | 48.18±3.1     |

**Table 2.** IC_{50} values of the compounds in different cell lines. The cells were treated with 0, 5,15,30, and 60 µM of each of P(3a), P(3b), P(3c), and P(3d) for 24 h. MTT assay was performed to calculate IC_{50} values. Values are mean of at least three different experiments and errors represent S.D. values.

| Cell line | Doses (µM) | 0  | 5  | 15 | 30 | 60 |
|-----------|------------|----|----|----|----|----|
| B16F10    | P(3a)      | 92 ±5.4 | 84 ±6.5 | 66 ±5.5 | 43 ±6.7 | 18 ±4.3 |
|           | P(3d)      | 86 ±5.4 | 75 ±6.5 | 64 ±5.5 | 38 ±6.7 | 12 ±4.3 |
| HCT 116   | P(3a)      | 84 ±5.7 | 76 ±6.4 | 58 ±5.7 | 39 ±6.2 | 19 ±6.2 |
|           | P(3d)      | 80 ±5.7 | 78 ±6.6 | 61 ±5.7 | 30 ±6.2 | 15 ±6.2 |
| MCF-7     | P(3a)      | 82 ±6.4 | 77 ±5.4 | 56 ±5.7 | 34 ±3.2 | 25 ±6.2 |
|           | P(3d)      | 84 ±6.3 | 69 ±5.4 | 63 ±5.7 | 35 ±3.2 | 21 ±6.2 |

**Table 3.** Percent of viable cells in P(3a) and P(3d) treated B16F10, HCT116 and MCF-7 cells. The cells were treated with 0, 5,15,30, and 60 µM of each of P(3a), and P(3d) for 24 h. Dead cells by trypan blue exclusion test and total number of cells (live and dead) were counted in different fields. Number of viable cells was calculated by subtracting the number of dead cells from total number of cells. Values are mean of at least three different experiments and errors represent S.D. values.
B16F10 cells through cell cycle arrest at G1 phase (Fig. 2B).

DNA fragmentation is one of the important events of cellular apoptosis. Treatment of P(3a) or P(3d) increased the level of fragmented DNA in B16F10 cells as seen in ELISA based cell death detection kit assay (Fig. 2C). DNA fragmentation pattern increased with dose. High level of DNA fragmentation was seen with 30 µM of either compound at 24 h. Level of DNA fragmentation was low with 60 µM of P(3a) and P(3d); possibly, a part of the cells undergo necrosis.

P(3a) and P(3d) induced apoptosis in B16F10 cells involves mitochondrial pathway

Cellular apoptosis normally involve intrinsic (mitochondrial) or extrinsic pathway or both. To examine the involvement of mitochondrial pathway, levels of caspase-9 and caspase-3, antiapoptotic Bcl-2, mitochondrial membrane potential were measured. Treatment with increasing concentrations of P(3a) and P(3d) for 24 h significantly increased the levels of caspase-9 and caspase-3, which were inhibited in the presence of their specific inhibitors Z-DEVD-FMK and Z-LEHD-FMK respectively (Fig. 3A, a,b).

To confirm the role of caspases in P(3a) and P(3d) induced apoptosis of B16F10 cells, percent cell death in presence of the caspase inhibitors was examined. Addition of Z-DEVD-FMK and Z-LEHD-FMK one hour prior to the challenge of P(3a) or P(3d) significantly lowered the percent cell death (Fig. 3A, c).

B16F10 cell death triggered by P(3a) and P(3d) activates caspase-9, which indicates the involvement of mitochondrial pathway. This was confirmed by measuring the status of cytosolic cytochrome c and the loss of mitochondrial membrane potential. Cytosolic cytochrome c level was increased and mitochondrial membrane potential was lowered with increasing concentrations of P(3a) and P(3d) as seen in 24 h of study (Fig. 3B, a,b).
The status of a number of pro and anti-apoptotic proteins regulating the mitochondrial pathway was altered during apoptosis. Treatment of P(3a) and P(3d) lowered the level of anti-apoptotic proteins Bcl-2, pro-caspase-3, pro-caspase-9, and full length PARP (Fig. 3C).

Effect of P(3a) on survival time or tumor growth in mice

P(3a) was injected i.p. in BALB/c mice for 24 h using doses of 5, 50 and 200 mg/kg. No visible sign of toxicity or mortality was noticed up to 200 mg/kg. Mice were injected with B16F10 cells subcutaneously and treated with 15 or 30 mg/kg/day of P(3a) on indicated days (Fig. 4A, a) by i.p. route. The survival rate of B16F10 injected mice remarkably increased following treatment with P(3a) compared to the untreated tumor bearing mice (Fig. 4A, b), while tumor volume was significantly decreased (Fig. 4A, c-e). Histological study revealed decreased microvascular density and mitotic index of the tumor cells (Fig. 4A, f,g).

Discussion

The aim of the present study was to find new chemical entities, capable of inducing apoptosis in cancer cells. We prepared the 3-(arylideneamino)-2-phenylquinazoline-4(3H)-ones derivatives P(3a), P(3b), P(3c) and P(3d) following the route described earlier [24]. Among the four derivatives, P(3a) and P(3d) showed higher cytotoxic activity on B16F10, and HCT-116, and relatively lower cytotoxic activity on the other cells tested here.

Compounds containing nitro group have been shown to possess various biological activities like antibacterial, insecticidal, anthelmentic etc. Flutamide, a
nitro group containing aromatic compound, is used in metastatic prostate cancer [31]. Nitro containing naphthalimide analogues showed increased cytotoxic activity in human melanoma cell [32]. Induction of apoptosis in HeLa and A549 cells by the nitroaromatic compound 1-chloro-2,4-dinitrobenzene (dinitrochlorobenzene, DNCB) has been reported [33]. As P(3a) contains a nitro-aromatic group in the 2-phenylquinazolinone ring system, its potent cytotoxic activity may be attributed to this group.

It is reported that the presence of hydroxyl group in the aromatic ring of a compound influences its biological activity [34]. Aromatic compounds containing methoxy group are also biologically active and they exhibit growth inhibition activity [35]. Compound P(3d) contains both hydroxy and methoxy groups in its quinazolinone ring. Presence of these two groups may be responsible for its effect on the cancer cell lines used. It may be pointed out that curcumin containing hydroxy and methoxy groups have been shown to possess higher cytotoxic activity on different cell lines [36].

Induction of apoptosis in the hyperproliferative cancer cells is one of the major targets of cancer chemotherapeutics. B16F10 is a highly metastatic cell line. Studies using B16F10 cell revealed that the two lead compounds P(3a) and P(3d) caused apoptotic like morphological and nuclear changes (Fig. 1B, a,b). DNA fragmentation and enhancement of annexin V positive cells in response to P(3a) and P(3d) challenge in B16F10 cells confirm the involvement of apoptosis. Also our study shows that P(3a) and P(3d) induced apoptosis of B16F10 cells proceeds through cell cycle arrest at G0/ G1 phase.

Curcumin was reported to induce apoptosis in melanoma cells through the mitochondrial pathway [37]. Our study shows that P(3a) and P(3d) induced death of B16F10 cells also involves mitochondrial pathway as reflected in loss of mitochondrial membrane potential, release of cytosolic cytochrome c, down regulation of Bcl-2, and up regulation of Bax and Bad. The involvement of an extrinsic pathway in P(3a) and P(3d) induced death of B16F10 cells is currently being tested by us.

The induction of apoptosis by nitro containing aromatic compounds in cancer cells is not well documented. However, our study shows that the nitro containing quinazolinone compound P(3a) also induces apoptosis in B16F10 cell through mitochondrial pathway. It is found that P(3a) potently
inhibit the B16F10 tumor growth and increase the survival rate of mice. The reduction in tumor growth is well correlated with the decrease microvascular density and mitotic index of the tumor cells. Most importantly, animals did not show any change in visual symptoms like hair loss weight loss, diarrhea and movement with doses 5, 50 or 200 mg/kg body weight.

In summary, we conclude that quinazolinone derivatives having a nitro group or both hydroxy and methoxy groups show significant anticancer activity on some specific cell lines such as B16F10, HCT 116 and MCF-7. Compounds P(3a) and P(3d) possibly follow mitochondrial pathway of apoptosis as seen in B16F10 cells.

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