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ORIGINAL PAPER

Synthesis, Crystal Structure, Cytotoxic and Apoptotic Activity of 2,4-Dichloro-6-methylquinoline on Human Oral Carcinoma Cell Line

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Abstract The present report describes the synthesis, IR spectra, 3-dimensional structure of the compound 2,4-dichloro-6-methylquinoline and evaluation of its anti-cancer activity using propidium iodide (PI) staining and annexin binding assay techniques. This derivative of quinoline was synthesized from the mixture of *p*-toluidine and malonic acid and synthesis has been achieved in a one-pot reaction from an aryl amine, malonic acid and phosphorous oxychloride. Crystallographic data reveals that the crystals belong to triclinic crystal system with space group *P-1* with the unit cell dimensions of $a = 7.14(1)$ Å, $b = 11.53(1)$ Å, $c = 11.97(1)$ Å and $\alpha = 90.18°$ (10), $\beta = 106.31°$ (10), $\gamma = 91.07°$ (10). The in vitro anti-cancer assay indicated that compound has cytotoxic and apoptotic activity on human oral squamous carcinoma (KB) cell line, thus it could be developed as a potent anti-cancer agent.

Keywords Aryl amine · Malonic acid · 2,4-Dichloro-6-methylquinoline · Crystal structure · Flow cytometry · Apoptosis

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Introduction

The increase in knowledge of cancer biology has lead to the development of several new classes of anti-cancer agents. Quinolines and quinilones are very important in medicinal chemistry because of their wide occurrence in several cytotoxic agents [1], natural products [2] and drugs [3]. These compounds affect mammalian cellular functions in vitro in several ways; at higher concentrations it inhibits DNA replication while, at lower concentration it may affect individual genes [4]. Recent studies have shown their anti-tumor activity in a variety of human tumor cell either through arresting the G1, G2/M phase of cell cycle and cyclin D kinase [5] or by down regulation of Bcl-2 leading to altered ratio of Bax-Bcl2 that favors apoptosis [6]. It has been seen that series of quinoline derivatives mainly halogen derivatives with nitrogen or amino group present in the aromatic ring exhibit excellent cytotoxic activity [7, 8] and hence are useful as anti-microbial and/or anticancer agents.

One of the major obstacles to effective cancer chemotherapy is the existence of multi drug resistance (MDR) proteins in cancer cells that expel xenobiotics from the cells via ATP dependent efflux pump [9]. To overcome MDR, enormous efforts have been made to find an inhibitor of the drug efflux pump and various compounds such as verapamil, cyclosporine, quinodine, tomoxifen, progesterone, reserpine, etc have been reported to overcome MDR in vitro [10] but these have not been successful due to their dose limiting toxicity. However, being natural product quinolines derivatives have shown promising use in MDR resistant tumor cells with lesser side effects. A clinically approved quinoline derivative drug called MS209 is used in Japan.

Although many preparative methods are available in the literature [11, 12], it is still challenging to explore new and simple synthetic methods for quinolines and quinolones; particularly for the highly functionalized ones [13–15]. The classical method of quinoline synthesis is Skraup's procedure [16]. To the best of our knowledge there is no simple and efficient method for the formation of functionalized quinolines. In this paper, we report the synthesis, IR, NMR, X-ray crystal structure of one of new derivative of quinoline and its anti-cancer activity on human oral squamous cell carcinoma cell line.

Materials and Methods

Synthesis of 2,4-Dichloro-6-methylquinoline (1)

A mixture of *p*-toludine (2.14 g, 0.02 mole), malonic acid (2.08 g, 0.02 mole) and phosphorous oxychloride (50 ml) was refluxed under dry condition for 15 h in a steam bath. The solution was cooled to room temperature and poured over crushed ice carefully and allowed to stand overnight. The solid settled was filtered to dryness and purified over a column of silica gel (60–120 mesh; 50 g) eluting with Petroleum Ether–benzene (4:1) to give 2,4-dichloro-6-methylquinoline (Fig. 1). The product was re-crystallized from benzene which melted at 95 °C in 72% yield.

Spectral Conformation of 2,4-Dichloro-6-methylquinoline (1)

In order to confirm the synthesis of the title compound IR spectra was recorded on Thermo Nicolet Model Avatar 330 FT-IR spectrometer using KBr disc.

^{1}H-NMR was recorded on Varian AMX (400 MHz) instrument using TMS as an internal reference and $CDCl_3$ as solvent. The mass spectra were recorded on Jeol JMS 300 mass spectrometer.

Crystal Structure Determination

2,4-Dichloro-6-methylquinoline was crystallized from its solution in 80:20 acetone–water mixture at room temperature (298 K) by slow evaporation. The unit cell parameters and X-ray intensity data were collected on CAD4 single crystal diffractometer with Cu Kα radiation. The unit cell parameters were refined by a least-squares fit of 25 high angle ($25 \leq \theta \leq 40°$) reflections. These reflections were centered individually on the diffractometer. Lorentz and polarization corrections were applied. The crystal dimensions were ($0.4 \times 0.3 \times 0.2$ mm^3). The structure was solved with direct methods using the program SHELXS-97 [17]. The coordinates of non-hydrogen atoms were refined anisotropically using program SHELXL-97 [18]. However, the temperature factors for hydrogen atoms were not refined. The final *R*-factor for 3,109 observed reflections [$I \geq 2\sigma(I)$] was 0.057. The atomic scattering factors used in these calculations were those of Cromer and Mann [19] for non-hydrogen atoms and Steward, Davidson and Simpson [20] for hydrogen atoms. The final atomic and positional coordinates are deposited to the Cambridge Crystallographic Data Centre with the deposition number **CCDC 606999**.

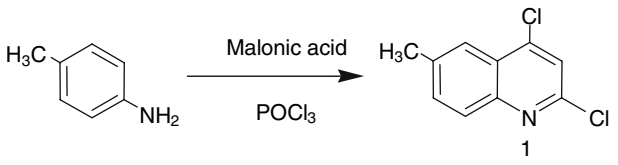

Fig. 1 Reaction scheme

Evaluation of Cytotoxic and Apoptotic Activity

The compound was dissolved in 1 ml of methanol, reconstituted in tissue culture media (RPMI 1640) and used in all the experiments. Control experiments were done with tissue culture media containing similar quantity of vehicle.

Flow Cytometry

Anti-tumor activity of the compound was assessed by flow cytometry on Human oral squamous cell carcinoma (KB) cell line (National Cell Science Centre, Pune, India) established in tissue culture. Tumor cell cytotoxicity was assayed by Propidium Iodide (PI) staining that intercalates with DNA of the dead cells.

Briefly, 2×10^6 tumor cells/ml was cultured in 24 well tissue culture plates in duplicate at 37 °C in CO_2 incubator with different concentrations (5, 10, 15, 20 and 25 µg/ml) of the compound for 1 h. The control wells received equal amount of tissue culture medium. The tumor cells were harvested after termination of the culture, washed twice with Phosphate Buffered Saline (PBS pH 7.2) and labeled with PI for 30 min at room temperature. At least 10,000 events were acquired in flow cytometer (BD LSR II, San Diego, California, USA) and the data was analyzed using FACS DIVA software. The results were expressed as percent cell survival.

Apoptotic activity of the compound was assessed by annexin binding assay using flow cytometry technique [21]. Briefly, the cells were treated with compound as

above. After harvesting, cells were washed twice with PBS (pH 7.2) and the cells pellet was treated with annexin V, FITC at room temperature for 45 min, washed twice with PBS and re-suspended in 1 ml of 1% paraformaldehyde. At least 10,000 events were acquired in flow cytometer. Frequency of apoptotic cells was calculated using FACS DIVA software and results were expressed as percentage. All experiments were conducted in triplicates.

Results and Discussion

Synthesis of Compound as a 2,4-Dichloro-6-methylquinoline

IR spectra recorded on Thermo Nicolet Model Avatar 330 FT-IR spectrometer using KBr disc showed absorptions at V_{max} = 1,650 cm^{-1} corresponding to (C=N), 1,060 cm^{-1} corresponding to (C–Cl) and 3,010 cm^{-1}(CH).

NMR was obtained in ppm: δ 2.5 (s, 3H, C_6–CH_3); δ 7.64–7.98 (m, 2H, C_7–H and C_8–H); δ 8.3 (s, 1H, C_5–H); δ 8.9 (s, 1H, C_3–H).

Mass spectra recorded a molecular ion peak at m/z 212 (M+), 214 (M + 2) and 216 (M + 4) confirmed the compound as 2,4-dichloro-6-methylquinoline.

Molecular Dimensions and Crystal Packing

The relevant crystal data of the compound, bond length and bond angles are given in Tables 1 and 2. The structure was solved by direct methods and refined by full matrix least-square. The final *R* factor was 0.057. An ORTEP plot of the molecule is shown in Fig. 2. The bond distances, N1–C2 and N1–C9, are shorter than the normally expected values and similar shortening is also observed in quinoline ring systems of structure like 8-hydroxyquinoline N-oxide [22], copper 8-hydroxyquinolinate [23] and zinc-hydroxyquinolinate dehydrate [24].

The low value for the bond length [C2a–Cl1a=], 1.738(3) Å and [C2b–Cl1b=], 1.737(2) Å and the exocyclic bond angle [N1b–C2b–Cl1b=], 116.47(1)° shows that the close packed bonding between N1, C2, and Cl1. Torsion angles: [C11–C6–C7–C8=], 179.61° for molecule a and 179.83° for molecule b and [C11–C6–C5–C10=], 179.43° for molecule a and 179.71° for molecule b indicates an extended conformation for the methyl group attached with the ring. The packing of the compound in unit cell is illustrated in Fig. 3. Hydrogen bond of type [C–H···Cl=], (2.77 Å) is observed in the crystal packing for the molecule [25].

Table 1 Crystal data and structure refinement for 2,4-dichloro-6-methylquinoline

Empirical formula	$C_{10}H_8Cl_2N$
Formula weight	213.07
Temperature	293(2) K
Radiation type	Cu Kα
Wavelength	1.54178 Å
Crystal system, space group	Triclinic, *P-1*
Unit cell dimensions	$a = 7.14$ (1) Å
	$b = 11.53$ (1) Å
	$c = 11.97$ (1) Å
	$\alpha = 90.18°$ (10)
	$\beta = 106.31°$ (10)
	$\gamma = 91.07°$ (10)
Volume	945.44(17) $Å^3$
Z, Calculated density	4, 1.49 mg/m^3
Absorption coefficient	5.736 mm^{-1}
Crystal size	.2 × .4 × .2 mm^3
θ range for data collection	3.83°–69.88°
Limiting indices	$-8 \le h \le 8$, $-14 \le k \le 14$, $0 \le l \le 14$
Reflections collected/unique	3,463/3,109
Completeness to $\theta = 69.88°$	82.6%
Refinement method	Full-matrix least-squares on F^2
Goodness-of-fit on F^2	1.070
Final *R* indices [$I > 2\sigma(I)$]	$R_1 = 0.057$, $wR_2 = 0.1704$
CCDC No	CCDC 606999

In-vitro-assay of Anti-cancer Activity

Cytotoxic effect of the compound was tested on human oral carcinoma (KB) cell line in 1 h culture at 37 °C and the results are shown in Fig 4. The tumor cells were treated with different doses (5, 10, 15, 20 and 25 μg/ml) of the compound and percent cytotoxicity was determined by Propidium Iodide uptake after termination of the culture. The compound showed 27% toxicity at 25 μg/ml dose 1 h of culture period. Time kinetics as determined using 25 μg/ml dose showed almost 40% cell death (cytotoxicity) at 4 h treatment (Fig 5). Apoptotic activity of a compound was assayed using optimum (25 μg/ml cytotoxic dose of the compound. Almost 60% of the tumor cells showed binding of annexin V at 4 h treatment period (Fig 6). The results suggest that 2,4-dichloro-6-methylquinoline is a potent anti tumor agent and it is able to induce apoptosis in the human oral carcinoma (KB) cells.

The exact mechanism of anti tumor activity of 2,4-dichloro-6,methylquinoline is not known. The previous studies reported that the substitution at C7 position of quinoline molecule by aliphatic group, OH or OCH_3 and by

Table 2 Bond lengths [Å] and angles [°] for 2,4-dichloro-6-methylquinoline

Cl(2A)–C(4A)	1.726(3)
Cl(1A)–C(2A)	1.738(3)
N(1A)–C(2A)	1.289(4)
N(1A)–C(9A)	1.362(3)
C(10A)–C(5A)	1.409(4)
C(10A)–C(4A)	1.414(4)
C(10A)–C(9A)	1.420(4)
C(7A)–C(8A)	1.367(4)
C(7A)–C(6A)	1.404(4)
C(6A)–C(5A)	1.366(4)
C(6A)–C(11A)	1.505(4)
C(9A)–C(8A)	1.409(4)
C(3A)–C(4A)	1.357(4)
C(3A)–C(2A)	1.398(4)
C(2A)–N(1A)–C(9A)	117.3(2)
C(5A)–C(10A)–C(4A)	125.0(2)
C(5A)–C(10A)–C(9A)	119.2(2)
C(4A)–C(10A)–C(9A)	115.8(2)
C(8A)–C(7A)–C(6A)	121.2(3)
C(5A)–C(6A)–C(7A)	119.0(2)
C(5A)–C(6A)–C(11A)	120.9(3)
C(7A)–C(6A)–C(11A)	120.1(3)
N(1A)–C(9A)–C(8A)	118.9(2)
N(1A)–C(9A)–C(10A)	122.8(2)
C(8A)–C(9A)–C(10A)	118.3(2)
C(7A)–C(8A)–C(9A)	120.8(3)
C(4A)–C(3A)–C(2A)	116.8(3)
C(6A)–C(5A)–C(10A)	121.5(2)
C(3A)–C(4A)–C(10A)	121.2(3)
C(3A)–C(4A)–Cl(2A)	119.3(2)
C(10A)–C(4A)–Cl(2A)	119.5(2)
N(1A)–C(2A)–C(3A)	126.2(3)
N(1A)–C(2A)–Cl(1A)	117.1(2)
C(3A)–C(2A)–Cl(1A)	116.8(2)
Cl(2B)–C(4B)	1.720(3)
Cl(1B)–C(2B)	1.737(3)
N(1B)–C(2B)	1.296(4)
N(1B)–C(9B)	1.363(4)
C(10B)–C(5B)	1.404(4)
C(10B)–C(9B)	1.411(4)
C(10B)–C(4B)	1.423(4)
C(8B)–C(7B)	1.361(5)
C(8B)–C(9B)	1.412(4)
C(11B)–C(6B)	1.501(4)
C(2B)–C(3B)	1.395(4)
C(6B)–C(5B)	1.374(4)
C(6B)–C(7B)	1.394(5)
C(3B)–C(4B)	1.352(4)
C(2B)–N(1B)–C(9B)	116.9(2)

Table 2 continued

C(5B)–C(10B)–C(9B)	119.8(2)
C(5B)–C(10B)–C(4B)	124.7(3)
C(9B)–C(10B)–C(4B)	115.4(2)
C(7B)–C(8B)–C(9B)	120.9(3)
N(1B)–C(2B)–C(3B)	125.9(3)
N(1B)–C(2B)–Cl(1B)	116.5(2)
C(3B)–C(2B)–Cl(1B)	117.6(2)
N(1B)–C(9B)–C(10B)	123.4(2)
N(1B)–C(9B)–C(8B)	118.7(2)
C(10B)–C(9B)–C(8B)	117.9(3)
C(5B)–C(6B)–C(7B)	119.2(3)
C(5B)–C(6B)–C(11B)	120.9(3)
C(7B)–C(6B)–C(11B)	119.9(3)
C(4B)–C(3B)–C(2B)	117.1(3)
C(3B)–C(4B)–C(10B)	121.2(2)
C(3B)–C(4B)–Cl(2B)	119.7(2)
C(10B)–C(4B)–Cl(2B)	119.1(2)
C(8B)–C(7B)–C(6B)	121.3(3)
C(6B)–C(5B)–C(10B)	120.8(3)

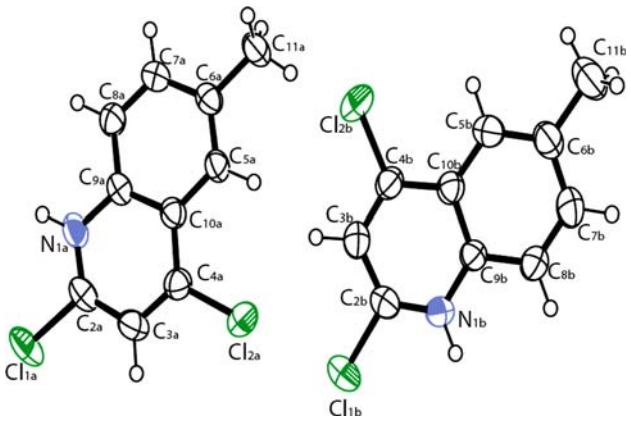

Fig. 2 Perspective view of the molecule 2,4-dichloro-6-methylquinoline at 50% ellipsoidal probability

the presence of halogens in the ring with Nitrogen inhibits DNA gyrase and topoisomerase during DNA replication. Such inhibition leads to the termination of DNA replication and eventually cell death [8, 26, 27]. Earlier studies show that 4-quinolones treatment down regulates Bcl-2 expression in tumor cells consequently leading to an altered ratio of Bax:Bcl-2. Such alteration triggers apoptotic cell death in the tumor cells 6. It seems reasonable to presume that substitution of aliphatic group at C6 position and chlorine at 2 and 4 positions may provide similar mechanism of action to the present compound.

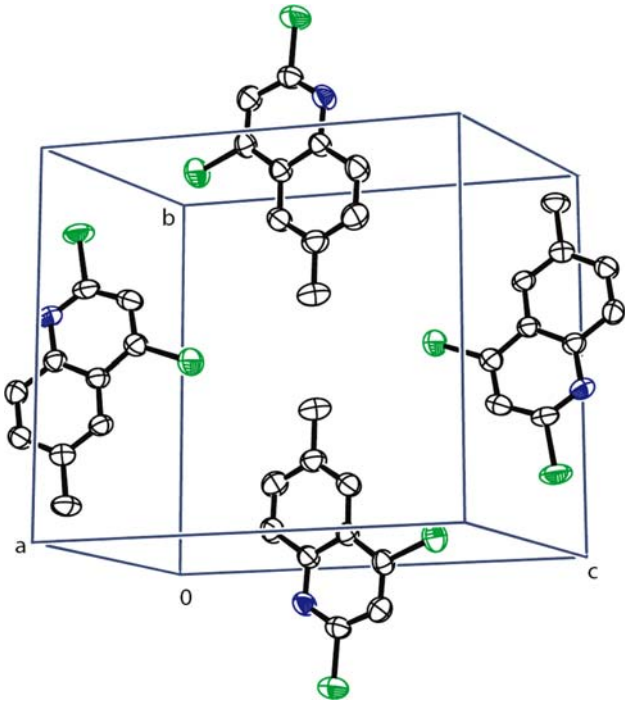

Fig. 3 Perspective view of the crystal packing of 2,4-dichloro-6-methylquinoline

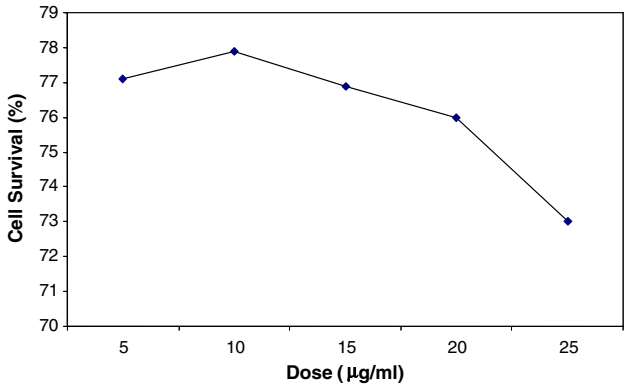

Fig. 4 In-vitro cytotoxic effect of different doses of quinoline on human oral carcinoma (KB) cell line. Tumor cell lines were pulsed with different concentrations of the PI and acquired in flow cytometer. The result represents averages of three experiments

Conclusions

The compound 2,4-dichloro-6,methylquinoline was synthesized, characterized and evaluated for the anti-cancer activity on oral carcinoma cell line. This compound contains aliphatic group at C6 position in quinoline molecule instead of C7 position and chlorine at 2 and 4 positions which are important for cytotoxic activity of eukaryotic cells. The cytotoxic activity of the quinolines with substitution at position 6 has been determined successfully for the first time. It seems that such substitution of aliphatic group at C6 position as well as halogens at 2 and 4 position

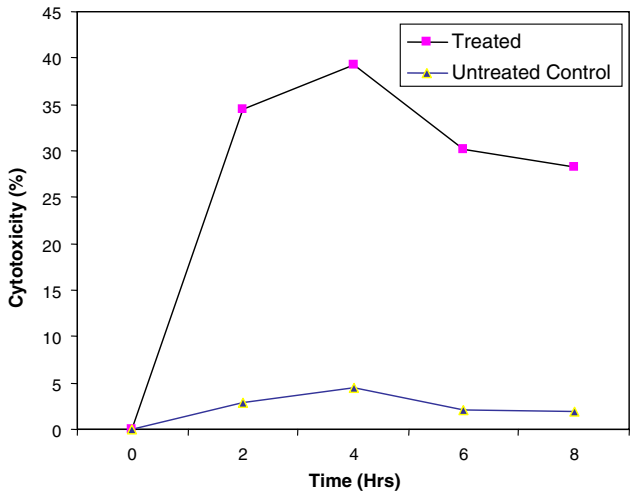

Fig. 5 In-vitro cytotoxicity of the quinoline on KB cell line. Tumor cell lines were pulsed with 25 µg/ml quinoline for 0, 2, 4, 6 and 8 h, labeled with PI and acquired in flow cytometer. The result represents averages of three experiments

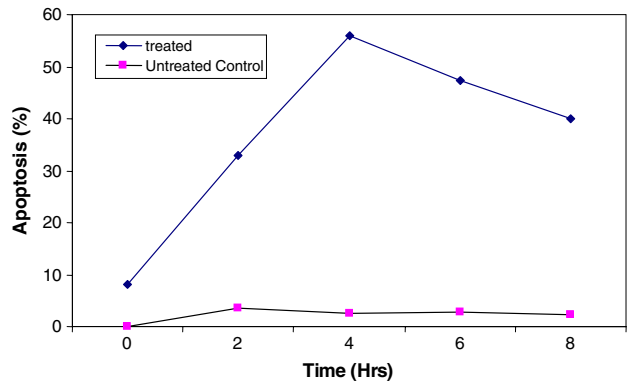

Fig. 6 In-vitro annexin binding assay showing frequency of apoptosis in tumor cell lines treated with 25 µg/ml quinoline for different time intervals. The cells were labeled with annexin V, FITC and acquired in flow cytometer. The results represent averages of three experiments

induces cytotoxicity and apoptosis in mammalian tumor cell line; hence series of compound with substitution at position 6 can be synthesized and tested for their anti-cancer activity.

Supplementary Material

CCDC (606999) contains the supplementary crystallographic data for this paper. These data can be obtained free of charge via http://www.ccdc.cam.ac.uk/conts/retrieving.html or from the Cambridge Crystallographic Data Centre, 12, Union Road, Cambridge CB2 1EZ, United Kingdom; Fax: (44) 1223-336-033; e-mail: deposit@cdc.cam.ac.uk

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