Synthesis and evaluation of selected 1,3,4-oxadiazole derivatives for in vitro cytotoxicity and in vivo anti-tumor activity

Amit Tiwari · N. Gopalan Kutty · Nitesh Kumar ·
Anil Chaudhary · P. Vasanth Raj · Rekha Shenoy ·
C. Mallikarjuna Rao

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Abstract The oxadiazole moiety is known for its anticancer activity through its antiangiogenic and mitostatic potential. Taking this as a cue, the present study was designed to investigate the anti-cancer potential of selected oxadiazole derivatives. Twelve 1,3,4-oxadiazole derivatives (AMK OX-1 to AMK OX-12) were synthesized and were tested for IC₅₀ values through brine shrimp lethality assay and MTT assay on HeLa and A549 cell lines. Four compounds, AMK OX-8, 9, 11 and 12 showed potential cytotoxicity activity with low IC₅₀ value. These compounds produced considerable cytotoxic effect on Hep-2 and A549 cancer cell lines. However, they were found to be comparatively safer to normal cell lines, viz., V-79 cell lines than to the tested cancer cell lines, such as HeLa, A 549, and Hep2 cell lines. The mechanism of

cytotoxicity was evaluated through nuclear staining and DNA ladder assay. Although DNA ladder assay showed DNA fragmentation (apoptotic phenomenon) in Hep-2 cells treated with only AMK OX-12, the staining procedures using acridine orange, ethidium bromide and propidium iodide showed apoptotic bodies in cells treated with AMK OX-8, 9 and 12 also. In JCI staining on isolated mitochondria of Hep2 cells, AMK OX-8, 9-11 and 12 displayed increasing fluorescence intensity with time which confirmed involvement of mitochondrial pathway and intrinsic pathway of apoptosis. All four compounds were found to be safe in acute oral toxicity study in Swiss albino mice. These derivatives were effective in reducing tumor size and weight in the in vivo DLA-induced solid tumor model. They were found to be significantly effective in reducing tumor volume and tumor weight.

A. Tiwari · N. Gopalan Kutty · N. Kumar ·
A. Chaudhary · R. Shenoy · C. Mallikarjuna Rao (✉)
Department of Pharmacology, Manipal College of
Pharmaceutical Sciences, Manipal University, Manipal,
Karnataka 576104, India
e-mail: mallikin123@gmail.com;
mallik.rao@manipal.edu

P. Vasanth Raj
Department of Pharmaceutical Biotechnology, Manipal
College of Pharmaceutical Sciences, Manipal University,
Manipal 576104, India

P. Vasanth Raj
Faculty of Pharmacy, AIMST University, Jalan Bedong
Semeling, 08100 Bedong, Kedah Darul Aman, Malaysia

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Introduction

Even though remarkable advances have been made in understanding cancer biology, the treatment of cancer remains an enigma. Cancer chemotherapeutic agents often provide short-term relief from the symptoms, with occasional cures. The effective doses of most of these therapeutic agents fall in the toxic range

(Remesh 2012). The drugs or their metabolites are highly reactive and produce unpleasant side effects by inducing varying degrees of cell destruction during treatment. Thus, search for new drugs is still on with tissue-specific cancer chemotherapy. Synthetic chemistry has always been a vital part in the highly integrated and multidisciplinary process of drug development. The approval process by US-FDA has shown most of the anticancer drugs are toxic, but those drugs are considered acceptable because a truly safe drug is not available (Basirian et al. 2013). Thus, because of the unavailability of safer and more specific anticancer drugs, continuous research in this area is required. The present study was aimed to identify such an anticancer drug with selective cytotoxicity towards cancerous cell. The selected 1,3,4-oxadiazole derivatives belong to one of such category of compounds, reported to possess antiangiogenic and antiproliferative activity (de Oliveira et al. 2012; Jain et al. 2013; Sankhe et al. 2015). The antiangiogenic and antiproliferative activities of compounds are mediated through down regulation of VEGF and inhibition of translocation of HIF-1 α . Derivatives of this group are also reported to be mitostatic agents with strong microtubule depolymerizing action (Jain et al. 2013; Kumar et al. 2009). Thus, considering 1,3,4-oxadiazole as potential moiety for anticancer drug discovery, the present work focused on evaluating selected 1,3,4-oxadiazole derivatives and studying their cytotoxicity at cellular (in vitro & in vivo) and, to some extent, at the molecular levels.

Materials and methods

Materials

Proteinase K, Acridine orange, ethidium bromide, propidium iodide and JC-1 mitochondrial stain were purchased from Sigma Aldrich, St. Louis, MO, USA. Methanol, acetone, sulfuric acid and other chemicals were procured from a reliable local vendor.

Test compounds

The 1,3,4-oxadiazole derivatives were synthesized through well-established synthetic steps as given in Fig. 1. Briefly, p-chlorophenol (0.1 M) was heated with monochloroacetic acid (0.1 M) in presence of

NaOH (0.2 M) solution (50 mL) in a beaker. The solution was heated to boil and then cooled. On acidification, p-chlorophenoxy acetic acid was obtained. The acid was then esterified with ethanol in presence of catalytic amounts of sulfuric acid. Ester was isolated and converted to its hydrazide by reacting with hydrazine hydrate in ethanol solution.

p-Chlorophenoxyacetic acid hydrazide (0.1 M) was refluxed with substituted aromatic aldehyde (0.1 M) in ethanol with catalytic amounts of glacial acetic acid for 10 min to get the respective Schiff Base. The Schiff base was then heated with acetic anhydride for 5 h to obtain the respective 2,5-disubstituted-1,3,4-oxadiazole. Twelve compounds, viz., AMK-OX-1 to AMK-OX-12 were synthesized (Fig. 1). The physicochemical properties of the compounds were recorded. Retardation factor (R_f) values for the compounds were evaluated using ethyl acetate:n-hexane (9.5:0.5) using TLC method. The synthesized compounds were characterized using FTIR (Shimadzu FTIR -8300), ^1H NMR and mass spectrometry data as follows:

4-{3-Acetyl-5-[(4-chlorophenoxy)methyl]-2,3-dihydro-1,3,4-oxadiazol-2-yl}phenyl acetate (AMK OX-1) molecular formula- $\text{C}_{19}\text{H}_{17}\text{O}_5\text{N}_2\text{Cl}$, predicted molecular weight— 388.75, melting point- 169–170 $^\circ\text{C}$, R_f 0.64, yield- 26 %, (IR KBr), 3261–3036 cm^{-1} (b) (–OH), 3200–3036 cm^{-1} (Ar), 1691 cm^{-1} (–NC=OCH₃), 1600 cm^{-1} (–C=N), 1274 cm^{-1} (–C–O–C-asymmetric), 1076 cm^{-1} (–C–O–C-symmetric).

1-{5-[(4-Chlorophenoxy)methyl]-2-(4-methylphenyl)-1,3,4-oxadiazol-3(2H)-yl}ethanone (AMK OX-2) molecular formula- $\text{C}_{18}\text{H}_{17}\text{O}_3\text{N}_2\text{Cl}$, Predicted molecular weight- 344.79, melting point- 182–184 $^\circ\text{C}$, R_f 0.76, yield- 17 %, (IR KBr), 3097–3182 cm^{-1} (Ar), 1678 cm^{-1} (–NC=OCH₃), 1587 cm^{-1} (–C=N), 1284 cm^{-1} (–C–O–C-asymmetric), 1068 cm^{-1} (–C–O–C-symmetric), 748 cm^{-1} (Ar–Cl), mass [M]: 344.

1-{5-[(4-Chlorophenoxy)methyl]-2-(4-chlorophenyl)-1,3,4-oxadiazol-3(2H)-yl}ethanone (AMK OX-3) molecular formula- $\text{C}_{17}\text{H}_{14}\text{O}_3\text{N}_2\text{Cl}_2$, predicted molecular weight- 365.21, melting point- 176–178 $^\circ\text{C}$, R_f 0.85, yield- 43 %, (IR KBr), 3064 cm^{-1} (Ar), 1678 cm^{-1} (–N–C=O–CH₃), 1585 cm^{-1} (–C=N), 1282 cm^{-1} (–C–O–C-asymmetric), 1058–1091 cm^{-1}

Fig. 1 Synthesis of 1,3,4-oxadiazole derivatives

(–C–O–C-symmetric), 731 cm^{-1} (Ar–Cl), mass $[M]^+$: 365.

1-{5-[(4-Chlorophenoxy)methyl]-2-(4-fluorophenyl)-1,3,4-oxadiazol-3(2H)-yl}ethanone (AMK OX-4) molecular formula- $\text{C}_{17}\text{H}_{14}\text{O}_3\text{N}_2\text{ClF}$, predicted molecular weight- 348.75, melting point- 194–196 °C, R_f - 0.80, yield- 28 %, (IR KBr), 3090 cm^{-1} (Ar), 1681 cm^{-1} (–N–C=O– CH_3), 1597 cm^{-1} (–C=N), 1290 cm^{-1} (–C–O–C-asymmetric), 1084 cm^{-1} (–C–O–C-symmetric), 750 cm^{-1} (Ar–Cl), 1230–1290 cm^{-1} (–C–F).

1-{5-[(4-Chlorophenoxy)methyl]-2-[4(dimethylamino)phenyl]-1,3,4-oxadiazol-3(2H)-yl}ethanone (AMK OX-5) molecular formula- $\text{C}_{19}\text{H}_{20}\text{O}_3\text{N}_3\text{Cl}$, predicted molecular weight- 373.83, melting point- 206–208 °C, R_f - 0.92, yield- 14 %, (IR KBr), 3076–3176 cm^{-1} (Ar), 1674 cm^{-1} (–NC=OCH₃), 1415–1604 cm^{-1} (–C=N), 1284 cm^{-1} (–C–O–C-asymmetric), 1064 cm^{-1} (–C–O–C-symmetric), 746 cm^{-1} (Ar–Cl).

1-{5-[(4-Chlorophenoxy)methyl]-2-(4-methoxyphenyl)-1,3,4-oxadiazol-3(2H)-yl}ethanone (AMK OX-6) molecular formula- $\text{C}_{18}\text{H}_{17}\text{O}_4\text{N}_2\text{Cl}$, predicted molecular weight- 360.79, melting point- 178–180 °C, R_f - 0.76, yield- 11 %, (IR KBr) 3055–3279 cm^{-1} (Ar), 1674 cm^{-1} (–NC=OCH₃), 1429–1579 cm^{-1} (–C=N), 1278 cm^{-1} (–C–O–C-asymmetric), 1068 cm^{-1} (–C–O–C-symmetric), 1165 cm^{-1} (–CO of –OCH₃).

1-{5-[(4-Chlorophenoxy)methyl]-2-[4-(trifluoromethyl)phenyl]-1,3,4-oxadiazol-3(2H)-yl}ethanone (AMK OX-7) molecular formula- $\text{C}_{18}\text{H}_{14}\text{O}_3\text{N}_2\text{ClF}_3$, predicted molecular weight- 398.76, melting point- 140–144 °C, R_f - 0.87, yield- 21 %, (IR KBr), 3072–3443 cm^{-1} (Ar), 1678 cm^{-1} (–NC=OCH₃), 1410–1579 cm^{-1} (–C=N), 1249 cm^{-1} (–C–O–C-asymmetric), 1057 cm^{-1} (–C–O–C-symmetric), 1014–1319 cm^{-1} (–C–F), mass spectroscopy $[M + 1]^+$: 400.

1-[2-(4-Bromophenyl)-5-[(4-chlorophenoxy)methyl]-1,3,4-oxadiazol-3(2H)-yl]ethanone (AMK OX-8) molecular formula- $\text{C}_{17}\text{H}_{14}\text{O}_3\text{N}_2\text{ClBr}$, predicted molecular weight- 409.66, melting point- 176–178 °C, R_f - 0.90, yield- 29 %, (IR KBr), 3066 cm^{-1} (Ar), 1676 cm^{-1} (–NC=OCH₃), 1408–1583 cm^{-1} (–C=N), 1249 cm^{-1} (–C–O–C-asymmetric), 1062 cm^{-1} (–C–O–C-symmetric), 520 cm^{-1} (–C–Br), mass $[M]^+$: 409.

1-{5-[(4-Chlorophenoxy)methyl]-2-(3-chlorophenyl)-1,3,4-oxadiazol-3(2H)-yl}ethanone (AMK OX-

9) molecular formula- $\text{C}_{17}\text{H}_{14}\text{O}_3\text{N}_2\text{Cl}_2$, predicted molecular weight- 365.21, melting point- 165–170 °C, R_f - 0.85, yield- 23 %, (IR KBr), 3093 cm^{-1} (Ar), 1685 cm^{-1} (–NC=OCH₃), 1404–1587 cm^{-1} (–C=N), 1257 cm^{-1} (–C–O–C-asymmetric), 1080 cm^{-1} (–C–O–C-symmetric), 732 cm^{-1} (Ar–Cl), mass $[M + 1]^+$: 367.

1-[2-(4-Aminophenyl)-5-[(4-chlorophenoxy)methyl]-1,3,4-oxadiazol-3(2H)-yl]ethanone (AMK OX-10) molecular formula- $\text{C}_{17}\text{H}_{16}\text{O}_3\text{N}_3\text{Cl}$, predicted molecular weight- 345.78, melting point- 198–200 °C, R_f - 0.30, yield- 9 %, (IR KBr) 3099 cm^{-1} (Ar), 3298–3416 cm^{-1} (–NH₂), 1672 cm^{-1} (–NC=OCH₃), 1406–1591 cm^{-1} (–C=N), 1273 cm^{-1} (–C–O–C-asymmetric), 1082 cm^{-1} (–C–O–C-symmetric).

3-{3-Acetyl-5-[(4-chlorophenoxy)methyl]-2,3-dihydro-1,3,4-oxadiazol-2-yl}phenyl acetate (AMK OX-11) molecular formula- $\text{C}_{19}\text{H}_{17}\text{O}_5\text{N}_2\text{Cl}$, predicted molecular weight- 388.75, melting point- 208–210 °C, R_f - 0.23, yield- 16 %, (IR KBr) 3281–3055 cm^{-1} (–OH), 3213–3155 cm^{-1} (Ar), 1676 cm^{-1} (–NC=OCH₃), 1427–1579 cm^{-1} (–C=N), 1280 cm^{-1} (–C–O–C-asymmetric), 1068 cm^{-1} (–C–O–C-symmetric).

2-{3-Acetyl-5-[(4-chlorophenoxy)methyl]-2,3-dihydro-1,3,4-oxadiazol-2-yl}phenyl acetate (AMK OX-12) molecular formula- $\text{C}_{19}\text{H}_{17}\text{O}_5\text{N}_2\text{Cl}$, predicted molecular weight- 388.75, melting point- 196–198 °C, R_f - 0.38, yield- 18 %, (IR KBr) 3103 cm^{-1} (Ar), 1763 cm^{-1} (–O–C=O–CH₃), 1697 cm^{-1} (–NC=OCH₃), 1429–1489 cm^{-1} (–C=N), 1290 cm^{-1} (–C–O–C-asymmetric), 1070 cm^{-1} (–C–O–C-symmetric), ¹H NMR (400 MHz, DMSO-d₆, δ , ppm): 2.32 (3H, s, –COCH₃), 2.36 (3H, s, –OCOCH₃), 6.96 (1H, s, O–CH–N), 7.03–7.95 (8H, m, Ar–H), mass $[M + 1]^+$: 390.

In vitro evaluation of anticancer activity

Brine shrimp lethality bioassay

Artemia salina (Brine shrimp) eggs were purchased from Brine Shrimp Direct (Ogden, UT, USA). The assay was carried out by using the procedure as described by Meyer et al. (1982) and Dhamija et al. (2013). Brine shrimp eggs were incubated at room temperature for 48 h for hatching. As the hatching occurred, ten nauplii were collected in 7 mL vials containing various concentrations of compounds

(5–20 µg/mL). The study was performed in triplicates for each dose level. Control vials were prepared by adding equal volumes of distilled water and maintained under illumination. After 24 h, surviving nauplii were counted using a magnifying glass (3×). The percentage deaths and LC₅₀ values were calculated by using Finney Computer program.

In vitro cytotoxicity in cultured cells by MTT assay

Cell lines were purchased from National Centre for Cell Sciences, Pune, India. HeLa (cervical carcinoma), Hep-2 (adult laryngeal carcinoma) and V-79 cells (Lung fibroblast) cultured in Dulbecco's Modified Eagle's Minimum (DMEM), A 549 (human lung carcinoma) cells cultured in DMEM/F-12 medium and Chang liver cells cultured in Minimum Essential Medium (Eagle) with Earle's BSS with non-essential amino acids and 1 mM sodium pyruvate. Media supplemented with 10 % heat-inactivated fetal bovine serum (FBS), 1 % antibiotic–antimycotic solution in a humidified incubator (5 % CO₂ in air at 37 °C). Cells (2×10^5 cells/ml) were cultured in T25 flasks and a stock cell suspension (1×10^5 cell/mL) was prepared. A 96-well flat bottom tissue culture plate was seeded with 50,000 cells in 0.1 mL of DMEM/MEM medium supplemented with 10 % FBS for 24 h in CO₂ incubator. Test compounds in 100 µL volume at 100, 50, 25, 12.5 µM concentrations in 0.2 % DMSO were added to the cells and incubated for 24, 48 or 72 h. The study was performed in triplicate for each dose level. The cells in the control group received only the medium containing 0.2 % DMSO. After the treatment, drug containing medium was removed, cells were washed with 200 µL of PBS, 100 µL of 1 mg/mL MTT reagent was added and incubated for 4 h at 37 °C. After 4 h of incubation, MTT was removed by inverting the plate and 200 µL of 100 % DMSO was added to solubilize formazan crystals. The optical density (O.D) was measured at 540 nm and expressed as percentage cell survival: (absorbance of treated wells/absorbance of control wells × 100) (Mosmann 1983).

In vivo anticancer screening

Animals

Swiss albino mice were acclimatized to the experimental room having temperature of 23 ± 2 °C,

controlled humidity conditions, and 12:12 h light: dark cycle. Animals were kept in polypropylene cages with two animals per cage. The mice were fed standard food pellets with access to water ad libitum. Study was conducted after approval from the Institutional Animal Ethics Committee of KMC, Manipal. No. IAEC/KMC/63/2009–2010.

Acute toxicity study

Acute toxicity assay was performed as per OECD guideline 425 in Swiss albino mice.

Solid tumor model

Selection of doses

Four test drugs, viz., AMK OX-8, AMK OX-9, AMK OX-11 and AMK OX-12, were selected for the in vivo study. The dose of each drug was arbitrarily kept at 100 mg/kg, which was <1/10 of the maximum tolerated dose determined through acute toxicity study. They were administered daily for 7 days from day one by intraperitoneal route. Cisplatin was used as standard drug at 3.5 mg/kg intraperitoneal dose.

Maintenance of cell lines

Dalton's lymphoma ascites cell lines (DLA), a spontaneous T-cell lymphoma of mouse origin were originally obtained from Amala Cancer Research Institute, Thrissur, India, maintained and propagated intraperitoneally by serial transplantation in adult male Swiss albino mice (Manjula et al. 2010).

Induction of solid tumor

The DLA-bearing mouse (donor) was taken 15 days after tumor transplantation. The ascitic fluid was drawn with an 18-gauge needle and sterile syringe. A small amount of ascitic fluid was tested for microbial contamination. Tumor viability was determined by trypan blue exclusion test. Cells in ascitic fluid were counted using hemocytometer and suitably diluted in saline to get a concentration of ten million cells per mL. This solution (0.1 mL) was injected intramuscularly to the right hind limb of male mice to obtain solid tumor. Treatment was given daily through intraperitoneal route for 7 days from day 1 of tumor inoculation (Kumar et al. 2014). Cisplatin

was injected on alternate days for 2 days. Tumor volume and tumor weight was monitored.

Tumor volume

The radii of developing tumor were measured using Vernier calipers, once every 3 days, for 1 month and tumor volume was calculated using the formula: $V = 4/3 \pi ab^2$, where a & b represented the major and minor radii, respectively.

Tumor weight

At the end of the fourth week the animals were sacrificed under anesthesia using diethyl ether, tumor was extirpated and weighed. The percentage inhibition was calculated with the formula: % Inhibition = $1 - B/A \times 100$, where A- the average tumor weight of control group, B-treated group.

Mechanistic studies

Nuclear staining

Staining methods were used to differentiate apoptotic cell death from necrotic cell death. The most commonly used stains are acridine orange, ethidium bromide and propidium iodide. For staining, cells were seeded in 6- well plate. After 24 h, medium was removed completely and selected drug concentrations were added and incubated for the next 24 h. Medium was removed, plate was washed with PBS and cells were fixed with 1 mL of 90 % methanol. The plate was kept at -20°C for 20 min. Methanol was removed and 1 mL of acetone was added and kept for 10 s. Acetone was removed and plate was washed with ice-cold PBS thrice. The desired stain (100 μL) was added and plate was incubated for 20 min. Then plate was washed thrice with PBS to remove excess dye and photographs were taken (Kumar et al. 2014).

DNA fragmentation (ladder) assay

The monolayer cell culture was trypsinized and adjusted to 1.0×10^5 cells/ml and seeded into 6- well plate. After 24 h, when a partial monolayer was formed, the supernatant was replaced with AMK OX 8, 9, 11 and 12 at their IC_{50} value and incubated

further for the next 48 h with 5 % CO_2 . Control wells were maintained with maintenance medium without any drug treatment. After 48 h the drug solutions in the wells were discarded, followed by DNA extraction. The cells were harvested by trypsinization and treated with 25 mM Tris-HCl buffer (pH 7.5) containing 0.5 % SDS, 0.5 mg/mL proteinase K and 5 mM EDTA at 55°C for 1 h. Further the cells were exposed to phenol: chloroform: isoamyl alcohol (25: 24: 1, v/v) followed by chloroform: isoamyl alcohol (24: 1, v/v) mixtures. 3 M sodium acetate (pH 5.2) with and absolute ethanol was used to precipitate DNA. After washing with PBS buffer, DNA was dried and re-suspended overnight at 37°C with 1 mM Tris-EDTA buffer (pH 7) containing 100 $\mu\text{g/mL}$ RNase A. DNA samples extracted from control cells and drug treated cells were subjected to agarose gel electrophoresis. The gel picture results were analysed using Alpha imager software (San Jose, CA, USA) (Kumar et al. 2014).

Mitochondria isolation and staining using JC-1 stain

Mitochondrial isolation and staining was performed according to supplier specifications of mitochondria isolation kit and isolated mitochondria staining kit using JC-1 stain from Sigma Aldrich, St. Louis, MO, USA. Monolayer of cell culture was trypsinised and cell count was adjusted to 1×10^6 cells/mL using growth medium. After 24 h when partial monolayer was formed, medium was discarded and cells were treated with drug dilutions prepared in maintenance medium. Control cells were treated with only maintenance medium. After 24 h of drug treatment cells were trypsinised and suspended in growth medium. Cell suspension was centrifuged at $600 \times g$ rpm for 5 min. After centrifugation supernatant was discarded and cell pellet was washed with ice cold PBS. Cells suspended in PBS were centrifuged again at $600 \times g$ rpm for 5 min at $20-80^\circ\text{C}$. The supernatant was discarded and cells were washed again with cold PBS. The cell pellet was resuspended in 0.5 mL of lysis buffer to get a uniform suspension and incubated on ice for 5 min with intermittent pipetting. Cell lysate was treated with 1 mL of extraction buffer and centrifuged at $600 \times g$ rpm for 10 min at $20-80^\circ\text{C}$. The supernatant was carefully transferred to a fresh tube and again centrifuged at $11,000 \times g$ rpm for

10 min at 40 °C. Supernatant was discarded carefully. The mitochondrial pellet was resuspended in 200 µL of storage buffer and stored in –20 °C until mitochondrial staining procedure was performed. Mitochondrial suspension was adjusted to 1000 µg/mL using storage buffer and stained with JC-1 (5,5',6,6'-tetrachloro-1,1'-3,3'-tetra ethyl benzimidazolocarbo-cyanine iodide) dye in 96 well plate. Each well loaded with 90 µL of JC-1 stain and mitochondrial suspension (10 µL) or storage buffer (10 µL) for the blank sample. Fluorescence was read at excitation wavelength of 490 nm and emission wave length of 590 nm with fluorescent micro plate reader. JC-1 aggregates in the intact mitochondria were visualized with help of fluorescent microscopy. FLU/mgP was calculated using the following formula:

$$\text{FLU/mgP} = \frac{(\Delta\text{FL}) \times \text{Dilution factor}}{V \times C}$$

where, FLU = fluorescence units, mgP = milligram protein, $\Delta\text{FL} = \text{FL}_{\text{sample}} - \text{FL}_{\text{blank}}$, Dilution Factor = dilution factor to prepare 1 mg/ml suspension, C = mgP/ml, V = volume of mitochondrial sample used for assay in ml, Dilution Factor = 0.5, C = 1 mg/mL, V = 0.01 mL. (Isolated mitochondria staining kit method from Sigma Aldrich (St. Louis, MO, USA)) (Sankhe et al. 2015).

Statistical analysis

Results were analyzed by one-way ANOVA followed by Tukey post hoc test using Prism-05 computer package (demo version).

Results

In vitro results

Brine shrimp lethality bioassay

The oxadiazole derivatives were screened by this assay in which nine compounds namely AMK OX-1, AMK OX-3, AMK OX-5, AMK OX-6, AMK OX-8, AMK OX-9, AMK OX-10, AMK OX-11, AMK OX-12 were found to be effective with IC₅₀ value <20 µg/mL (Table 1).

In vitro cytotoxicity assay

Since Brine Shrimp lethality test used in the study was a preliminary assay for testing cytotoxicity, cancer lines were also used to test these compounds. Percentage cytotoxicity was evaluated after incubation for 48 h on two cell lines viz., HeLa and A549. On A549, four compounds, viz., AMK OX-8, AMK OX-9, AMK OX-11 and AMK OX-12 showed potent cytotoxicity with IC₅₀ value <50 µM i.e., 25.04, 20.73, 45.11, and 41.92 µM, respectively. On HeLa cell line, three compounds, viz., AMK OX-8, AMK OX-10 and AMK OX-12 showed potent cytotoxicity with IC₅₀ value <50 µM i.e., 35.29, 5.34, 32.91 µM respectively (Table 1).

Based on MTT assay and Brine Shrimp Lethality assay, four compounds from the series of twelve oxadiazole compounds were selected for further evaluation, viz., AMK OX-8, 9, 11 and 12.

In vitro cytotoxicity of selected compounds

Short-term and long-term cytotoxicity was assessed for these four selected compounds at 24 and 72 h on HeLa and Hep-2 to assess time-dependent cytotoxicity. On HeLa cell line, AMK OX-8 showed maximum cytotoxicity after 48 h incubation, while AMK OX-11 and 12 showed maximum cytotoxicity after 24 h of incubation (Table 2). In fact, the cytotoxicity of AMK OX-11 and 12 decreased with time (IC₅₀- 11.26, 32.91 and 71.74 µM and 42.11, 116.08 and 243.3 µM at 24, 48, and 72 h, respectively, for OX11 and OX12). AMK OX-9 showed IC₅₀ value beyond 100 µM at all tested time points on Hep2 cells.

On Hep-2 cell line, the cytotoxicity of AMK OX-8, OX-9 and OX-12 increased with time. AMK OX-12 showed maximum cytotoxicity at 72 h among all tested compounds (IC₅₀- 0.0007 µM at 72 h) (Table 2).

In vitro MTT assay on normal cell lines (Chang liver and V79)

This study was performed to assess whether compounds were cytotoxic to normal cell lines. On Chang liver cell line, a subclone of HeLa, only AMK OX-11 showed IC₅₀ value of more than 100 µM after 72 h.

Table 1 Cytotoxicity of compounds

Compounds	Brine shrimp lethality assay IC ₅₀ (μM)	MTT assay on A549 cell line IC ₅₀ (μM) after 48 h	MTT assay on HeLa cell line IC ₅₀ (μM) after 48 h
AMK OX-1	18.87	65.34	81.2
AMK OX-2	50.65	135.58	174.5
AMK OX-3	0.807	94.55	114.86
AMK OX-4	41.15	275.85	436.84
AMK OX-5	19.96	210.65	114.86
AMK OX-6	18.29	5.94	87.88
AMK OX-7	>500	74.89	120.09
AMK OX-8	1.7	25.04	35.29
AMK OX-9	1.72	20.73	66.47
AMK OX-10	8.5	32.02	5.34
AMK OX-11	1.6	45.11	32.91
AMK OX-12	0.58	41.92	116.08
Standard (Doxorubicin)	3.5	8.75	11.12

Table 2 Evaluation of cytotoxic effect of selected oxadiazoles through MTT assay

Compounds/cell lines/time (h)	Cancer cell lines						Normal cell lines					
	IC ₅₀ (μM) on HeLa			IC ₅₀ (μM) on Hep2			IC ₅₀ (μM) on Chang liver			IC ₅₀ (μM) on V 79		
	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
AMK OX-8	42.09	35.29	38	62.97	42.83	49.97	320.59	87.87	54.05	196.12	599.7	136.78
AMK OX-9	133.33	113.1	105.95	82.99	82.94	44.57	>400	246.24	39.73	89.38	706.86	177.37
AMK OX-11	11.26	32.91	71.74	185.71	60.9	46.62	246.75	305.01	122.83	215.5	224.97	401.16
AMK OX-12	42.11	76.34	243.3	92.9	29.11	0.0007	180.76	169.2	54.45	293.32	169.2	80.68
Doxorubicin	19.25	11.12	8.2	22.49	27.85	14.87	–	–	–	–	–	–

On V-79 cell line except for AMK-OX12 the other compounds showed IC₅₀ values of more than 100 μM after 72 h. All the four oxadiazoles have high IC₅₀ values on both normal cell lines compared to cancerous cell lines at each tested time points, indicating that they have low toxic effects on normal cells (Table 2).

In vivo results

Acute toxicity study

None of the four tested compounds (AMK OX-8, 9, 11, 12) produced any toxicity up to 2000 mg/kg *p.o.* in Swiss albino mice.

Solid tumor model

Effect of compounds on tumor volume

Cisplatin significantly ($p < 0.05$) reduced tumor volume compared to control animals. On every monitored day, volume reduction was observed. AMK OX-8, 11 and 12 significantly ($p < 0.05$) reduced tumor volume on the 20th, 25th, and 30th day. AMK OX-9 significantly ($p < 0.05$) reduced tumor volume on the 5th and the 30th day. In the case of AMK OX-11, there was significant ($p < 0.05$) reduction in tumor volume on the 15th day also. Thus, it might be more potent than the other test compounds (Fig. 2; Table 3).

Effect of compounds on tumor weight

On day 30, tumor weight was found to be 4.4 g in control mice. Significant ($p < 0.05$) reduction in tumor weight was observed in cisplatin-treated mice (1.02 g). The 4 selected oxadiazoles (AMK OX-8, 9, 11 and 12) also significantly ($p < 0.05$) reduced tumor weight (Fig. 3).

Mechanistic studies

Nuclear staining

Nucleomorphological changes are important features seen after the treatment of cell with test compounds. In the present study, three stains were used to study the

nucleomorphological changes after treatment of cells with AMK OX-8, 9, 11 and 12. Stained images showed typical apoptotic morphology such as nuclear fragmentation and cytoplasm shrinkage in case of Hep-2 cells treated with AMK OX-8, AMK OX-9, AMK OX-12 (Fig. 4).

DNA ladder assay

Positive conclusion of nuclear staining observation led to further analysis of AMK OX 8, 9, 11 and 12 for their DNA fragmentation study. Control DNA in lane number 1, showed intact single band of DNA. Samples from lane number 2–5 were DNA samples of cells treated with AMK OX 8, 9, 11 and 12, respectively. Drug samples AMK OX 8 and 9 (lane number 2 and 3) showed mild damage whereas significant DNA damage was observed in lanes 4 and 5, where the cells have been treated with AMK OX 11 and 12 (Fig. 5). Thus

Fig. 2 Effect of compounds on tumor volume in DLA inoculated mice. Legend: the values are mean $\pm$ SEM of six animals

Fig. 3 Effect of selected oxadiazoles on tumor weight in mice inoculated with DLA. Legend: the values are mean $\pm$ SEM of six animals where ^a $p < 0.05$ versus control

Table 3 Effect of selected oxadiazole derivatives on tumor volume in mice injected with DLA cells

Groups/day	Tumor volume (cm ³) mean ± SEM						
	0	5	10	15	20	25	30
Control	0.06 ± 0.01	0.09 ± 0.01	0.16 ± 0.01	0.22 ± 0.018	0.31 ± 0.02	0.53 ± 0.08	0.81 ± 0.12
Standard (Cisplatin)	0.02 ± 0.00 ^a	0.025 ± 0.00 ^a	0.05 ± 0.00 ^a	0.12 ± 0.01 ^a	0.11 ± 0.01 ^a	0.08 ± 0.01 ^a	0.087 ± 0.01 ^a
AMK OX-8	0.03 ± 0.00 ^a	0.07 ± 0.00	0.12 ± 0.01	0.18 ± 0.02	0.18 ± 0.03 ^a	0.22 ± 0.03 ^a	0.32 ± 0.04 ^a
AMK OX-9	0.03 ± 0.00 ^a	0.05 ± 0.00 ^a	0.11 ± 0.01	0.17 ± 0.02	0.23 ± 0.02	0.37 ± 0.07	0.45 ± 0.08 ^a
AMK OX-11	0.04 ± 0.00 ^a	0.09 ± 0.01	0.13 ± 0.01	0.12 ± 0.01 ^a	0.17 ± 0.01 ^a	0.24 ± 0.03 ^a	0.46 ± 0.06 ^a
AMK OX-12	0.03 ± 0.00 ^a	0.08 ± 0.00	0.12 ± 0.04	0.18 ± 0.02	0.21 ± 0.03 ^a	0.29 ± 0.05 ^a	0.28 ± 0.05 ^a

All values are means $\pm$ SEM of six samples where ^a $p < 0.05$ versus control

Fig. 4 Nuclear staining using various stain on Hep-2 cells. Legend: all the compounds were added at their IC₅₀ on Hep-2 cell line. The *arrows* indicate nucleomorphological changes i.e., condensed nuclei and shrinkage of cytoplasm

the DNA fragmentation result confirms the anticancer properties of AMK OX 11 and 12.

Mitochondria isolation and staining using JC-1 stain

In this assay an effective distinction between apoptotic and healthy cells was checked with the help of fluorochrome JC-1 dye by measuring fluorescent intensity. Cisplatin was used as a standard anticancer drug. Valinomycin was used as a positive control for mitochondrial opening. As displayed in Fig. 6, untreated cells (negative control) showed less fluorescence intensity. AMK OX-8 and cisplatin treated cells showed high fluorescence intensity in comparison with untreated control cells. Among all the compounds tested, AMK OX-8 showed maximum fluorescent intensity followed by AMK OX-9, AMK OX-11 and AMK OX-12 respectively (Fig. 6).

Discussion

The present study was aimed at investigating in vitro and in vivo anticancer activities of selected oxadiazole derivatives synthesized in our lab. Preliminary cytotoxic screening was performed using brine shrimp lethality bioassay, which gave initial information about cytotoxic nature of test drugs. This assay is one of the preliminary studies to evaluate cytotoxic nature of the compounds, where the compounds' ability to prevent the growth of rapidly dividing cells could be evaluated (Genupur et al. 2006). Compounds AMK OX-1, AMK OX-3, AMK OX-5, AMK OX-6, AMK OX-8, AMK OX-9, AMK OX-10, AMK OX-11, AMK OX-12 were found to be cytotoxic in this assay. The cytotoxic potency was further confirmed by MTT assay against HeLa and Hep-2. In MTT assay on the Hep-2 cell line, AMK OX-8 and AMK OX-12 showed better activity, particularly at 48 h compared with the

Fig. 5 Apoptosis detection by DNA fragmentation assay on Hep-2 Cell lines (48 h study). Legend: the compounds were added at their respective IC_{50} on Hep-2 cell line. DNA fragmentation studies of AMK OX 8, 9, 11 and 12 against cancer cells by using agarose gel electrophoresis. Lane M: Marker DNA ladder 1 kb (Lane M marker DNA lane separately performed and inserted for reference); Lane 1: control cells (untreated); Lane 2: AMK OX 8 treated; Lane 3: AMK OX 9 treated; Lane 4: AMK OX 11 treated; Lane 5: AMK OX 12 treated

rest of selected derivatives. On HeLa cell line, AMK OX-8 showed more elevated cytotoxicity at 24 h and AMK OX-11 at 24, 48 h and AMK OX-12 at 24 h. In the 48 h study on A549 cell line, AMK OX-8 and AMK OX-9 were found to be effective with IC_{50} value 25.04 and 20.73 μ M respectively.

Our study revealed that out of the 12 oxadiazole derivatives, four had the potential to reduce cancer cell growth. AMK OX-8 and AMK OX-12 were most effective in reducing cancer cell growth in in vitro studies. The safety of the compounds was evaluated on two normal cell lines, namely, Chang liver and V-79 cell lines. In the MTT assay on normal cell lines (Chang and V-79), the four derivatives AMK OX-8, AMK OX-9, AMK OX-11, AMK OX-12 showed only limited cytotoxicity after 24, 48 and 72 h exposure compared to cancer cells. The cytotoxicity of compounds was expected to increase with time, while decrease might reflect instability in the solution or development of resistance to the cancer cells for the tested compound or influence of variables such as number of cells seeded, incubation time, concentration of serum etc. Although, AMK OX-11 and AMK OX-

Fig. 6 Effect of selected oxadiazoles on the mitochondrial membrane potential. Legend: Hep2 cells were treated with AMK OX-9, 10, 11 and 12 at their IC_{50} value. After 24 h mitochondrial fraction was separated and stained with JC-1. Change in fluorescence intensity with respect to time was recorded. Data indicate kinetic study showing changes in fluorescence units and fluorescence intensity in Hep2 cells

12 showed increase in IC_{50} on HeLa cells value over the time (from 24 to 72 h), the remaining treatments showed time dependent decrease in IC_{50} value. The compounds exhibited specific toxicity towards cancer cells and were found to be safe towards normal cells with higher IC_{50} value except on Chang liver cells, where compounds AMK OX-8, AMK OX-9, AMK OX-12 showed less than 100 μ M IC_{50} at 72 h. This might be due to the contaminant characteristic of Chang liver cells with HeLa, which was originally considered to be derived from normal human liver tissue. AMK OX-11 showed specific toxicity only towards cancer cells at the same time found to be safe against normal cells with higher IC_{50} value (more than 100 μ M). All these four derivatives (AMK OX-8, AMK OX-9, AMK OX-11, and AMK OX-12) were also found to be safe in acute oral toxicity study at 2000 mg/kg *p.o.* in Swiss albino mice. This indicates that the compounds do not have any major toxicity towards normal cells. Ahmad et al. (2014), have reported similar activity of the synthesized compounds specifically cytotoxic towards cancer cells only and safe towards normal cells (Ahmad et al. 2014).

For assessing efficacy of the selected oxadiazoles, DLA-induced solid tumor model was used. After tumor transplantation, regular and rapid growth in

tumor was assessed using Vernier calipers. The four selected derivatives (AMK OX-8, AMK OX-9, AMK OX-11, and AMK OX-12) significantly reduced tumor volume and tumor weight. Therefore, inhibition of tumor progression and development supports their anticancer activity.

There are several ways through which anticancer drugs control growth of malignant cells at molecular and cellular levels. Broadly, they may act by reducing the expression of positive cell cycle regulators, causing over expression of negative cell cycle regulators, reducing the tumor vascularity and, inducing cell death either by necrosis or by promoting apoptosis (Lee and Yang 2001). The available experimental data are not sufficient to pinpoint the possible mechanism of action of the active compounds. However, there is substantial evidence in the present study to indicate that the compounds promoted apoptosis or programmed cell death. Four out of the 12 synthesized compounds showed the potential of cytotoxicity and effective cell growth reduction. Potency of the drugs depends on the nature of the structure. Since MTT method determination is dependent on the synthesis of mitochondrial succinate dehydrogenase enzyme and the mitochondrial activity, the anticancer effect of the compounds could be possibly due to intrinsic pathway mechanisms (Sankhe et al. 2015). Also similar papers (Ahmad et al. 2014; Sankhe et al. 2015) explain the possibility of the compounds exhibiting anticancer activity by altering the mitochondrial stability. This would lead to a cascade of reactions involving cytochrome c, resulting in apoptosis. Our result also confirms the DNA damage by nuclear staining and DNA ladder assay. The DNA ladder assay conducted on Hep-2 cell lines showed that two active compounds viz., AMK OX-11 and AMK OX-12, produced fragmentation of DNA (apoptotic phenomenon) by ladder formation. Ladder formation is the marker of apoptotic phenomenon (Meikle et al. 1996). The chemical nature of oxadiazoles would have to be suspected as causing DNA-damage leading to apoptosis induction in cancer cell lines. The staining studies further confirmed the induction of apoptosis, marked by the presence of apoptotic bodies in the cells that were treated with the four compounds.

Mitochondria are important organelles to evaluate apoptotic signals. Early event in apoptosis is marked

by dissipation of mitochondrial membrane potential which can be measured at the inner membrane level by observing the uptake of the cationic carbocyanine dye JC-1 (5,5',6,6'-tetrachloro-1,1'-3,3'-tetra benzimidazolocarbocyanine iodide) into the mitochondrial matrix. Any dissipation of the mitochondrial membrane potential prevents the accumulation of the JC-1 dye in the mitochondria and thus the dye is dispersed in the cytoplasm shifting from red (agglomerated JC-1) to green fluorescence (JC-1 monomers) (Plášek and Sigler 1996). A polarized mitochondrial membrane draws additional dye into the mitochondrial membrane from the medium and reduces the fluorescence intensity of the suspension. Whereas if there is depolarization or any damage to the mitochondrial membrane potential dyes is released dye into the medium, increasing the overall fluorescence (Perelman et al. 2012). Our results confirmed the anticancer potential of the tested compounds on Hep2 cells by increasing the fluorescence intensity, which was a direct confirmation of the mitochondrial damage and change in the mitochondrial membrane potential as the possible mechanism for the anticancer property of the tested compounds. Any change in mitochondrial membrane potential leads to cytochrome c release followed by apoptosis (Ahmad et al. 2014; Sankhe et al. 2015). Thus the results strongly indicated the biological basis for supporting anticancer effect which might be involving mitochondrial pathway and intrinsic pathway of apoptosis and anti-cancer activity.

Conclusion

Thus it can be concluded that the oxadiazole derivatives possibly possess significant antitumor activity in both in vitro and in vivo. Among the tested compounds, AMK OX-8, AMK OX-11 and AMK OX-12 were the most effective anticancer agents by inducing apoptosis in cancer cells. Though some derivatives showed promising results, more data are required to know their exact potential as candidate molecules for cancer chemotherapy.

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