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Evaluation Of Apoptosis Inducing Activity Of An Oxadiazole Based Potential Anticancer Compound

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Abstract: Apoptosis, a programmed cell death, removes the unwanted old and faulty cells. It is well known that majority of anticancer agents also exhibit apoptosis induction. In continuation of our interest in search of newer anticancer compounds, we evaluated the *in vitro* anticancer activity of some substituted 1,3,4-oxadiazoles. To our surprise, compound **1,** exhibited good anticancer activity against majority of the cell lines in the panel of 60 cell lines tested at NCI, US. This prompted us to evaluate the apoptosis inducing potential of this compound. We herein report the evaluation of apoptosis inducing activity of compound **1** against K-562, MCF-7, HCT-116, MOLT-4 cancer cell lines using flow cytometer. This compound showed promising activity against K-562 cell line (45%), which was further confirmed by laser confocal microscopy. There was a good correlation between the *in vitro* anticancer activity and proapoptic activity.
Keywords: Apoptosis**;** Flow cytometry; Confocal microscopy.

Introduction:

Oxadiazole is one of the biologically important classes of heterocyclic compounds since it exhibits wide range of activities like antimicrobial, antiepileptic, anti- inflammatory, anti-cancer etc. [1-4]. Recently a group of scientist from Maxim pharmaceuticals reported the anticancer activity of some substituted oxadiazole[5]. 4-Thiazolidinones is another heterocyclic moiety of special pharmacological interest because of its wide spectrum of activities [6-8] including anti-cancer activity.

Apoptosis a programmed cell death is the natural process of homeostasis. Process of apoptosis removes the unwanted, old and faulty cells [9]. Thus, in malignancy the process of apoptosis occurs at the rate slower than the normal; hence a drug, which could induce apoptosis, is required to control the unwanted cell growth [10, 11]. It is well proven that many anticancer agents also induce apoptosis [12]. A variety of synthetic heterocyclic compounds have been reported for their apoptosis inducing ability [13]. In search of some newer anticancer agents, we synthesized a series of compounds comprising of 1, 3, 4-oxadiazole and 4-

thazolidinone ring systems and analyzed their *in vitro* anticancer activity. To our surprise one of the compounds, compound **1,** was found active against majority of the cell lines from the panel of 60 cell lines of NCI, USA [14]. This prompted us to evaluate the apoptosis inducing potential of the compound **1**.

Apoptosis can be detected by number of techniques like cell cycle analysis using flow cytometer, nuclear fragmentation by laser confocal microscopy, etc. [15]. Confocal laser scanning microscopy is a powerful tool for both morphological analysis and macromolecular localization. Thus, condensed chromatin and nuclear fra gmentation are easily determined by studying the optical sections of cells stained with dyes like DAPI, propidium iodide (PI). This technique is primarily used for qualitative determination of apoptosis.

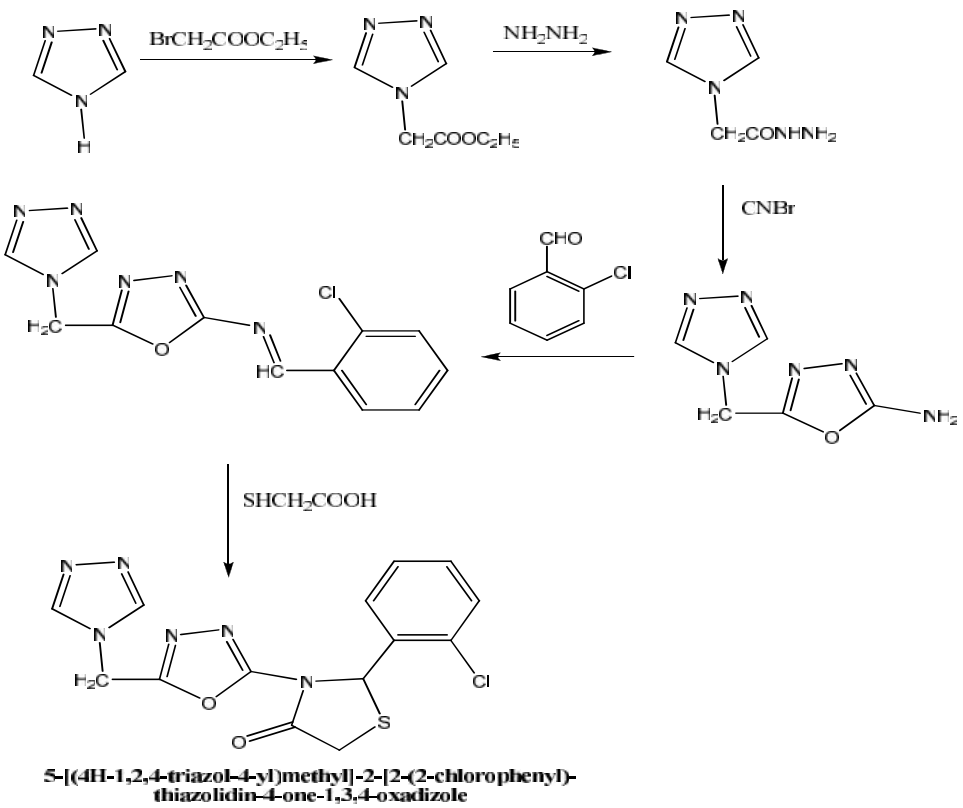
In the present study we evaluated apoptosis inducing activity of compound **1** against four cell lines K-562 (Leukemia), MCF-7 (Breast Cancer), HCT-116 (Colon cancer), MOLT-4 (Leukemia). These cell lines are the representatives of some most prevalent cancers like leukemia, colon and breast cancer. It was observed in the literature that oxadiazole backbone has potential anti-cancer activity against few of these cancers. Infact these compounds exhibit their effect via induction of apoptosis. Hence these cell lines were specifically selected in this study.

Materials and Methods:

Chemical and reagents:

All fine chemicals and specialized chemicals like PI, RNase and DAPI were obtained from Sigma Chemicals, India. All reagents were prepared using deionised (Millipore) water.

Scheme of synthesis of Compound 1



Compound **1**

Anti-cancer activity protocol:

Synthesis of compound **1** and its anticancer activity is already reported by the authors [14]. Anticancer assay of Compound **1** was performed according to the US NCI protocol [16]. The cytotoxic and/or growth inhibitory effects of the various compounds synthesized by us reported elsewhere [14] was tested *in vitro* at the concentration of 10^{-5} M against the full panel of nearly 60 human tumour cell lines derived from nine neoplastic diseases namely lung, colon, breast, ovarian, leukaemia, renal, melanoma, prostate and CNS. A 48 hour continuous drug exposure protocol was followed and an SRB protein assay was used to estimate cell viability or growth. This number is growth relative to the no-drug control and relative to the time zero number of cells. This allows detection of both growth inhibition (values 0 to 100) and lethality (values less than 0). A value of 100 means no growth inhibition, a value of 40 means 60% growth inhibition, a value of 0 means no net growth over the course of the experiment, a value of -40 means 40% lethality and a value of -100 means all cells are dead. The *in vitro* anticancer activity against the full panel of nearly 60 human tumour cell lines is reported elsewhere**.** Amongst the series of 10 oxadiazole derivatives synthesized, Compound **1** showed excellent anti-cancer activity in majority of the cell lines (data not shown). [14].

Hence, in the present work compound **1** was tested for its apoptosis inducing potential using quantitative flow cytometry and qualitative confocal laser microscopy.

Flow cytometric analysis:

Cell lines like K562, MOLT-4, MCF7 and HCT116 were used. Cells were distributed in 24 well plates at concentration of 0.2 X 106 cells / well / 1 mL and they were incubated at 37^0C for 24 h. Then the compound was added to the cells at different concentrations i.e. 0.25, 0.5, 1, 10, 50 µg/mL. After 48 h cells were harvested and washed with 1X PBS (phosphate buffer solution) and fixed with 70% ethanol (chilled) and stored at 4^0C. On the day of acquisition, they were washed with 1X PBS. The cells were suspended in 500µL of 1X PBS, 100 µL of RNase A (1mg/mL- stock) and 100 µL PI (400 µg/mL- stock), and incubated at 37^0C for 30 min. The fluorescence detected at FL-2 filter (585 nm) and induction of apoptosis was determined by DNA content analysis (sub-G1) through flow cytometry using PI. The cells were acquired at FACS Calibur (Becton Dickinson) flow cytometer and analyzed.

To confirm the apoptosis, changes in DNA content were analyzed. The profile of PI stained events clearly distinguished nuclei with normal diploid DNA in control cells from the nuclei with hypo diploid DNA found in treated cells.

Confocal microscopy:

In the present study, chromatin condensation and nuclear fragmentation was visualized by confocal microscopy using DAPI staining. Briefly, cultured cells (K -562 and MCF-7, $1x10^6$ cells) were harvested and fixed with 500µL 1% paraformaldehyde. They were incubated for 15 minutes in ice and washed with 5mL of PBS and centrifuged at the speed of 1000 rpm for 10 minutes. Cells were then permeabilised using saponin buffer and stained with DAPI for 3 minutes. This was followed by washes with PBS and finally cells were mounted for confocal microscopy. Cells were analysed on a LSM 510 laser confocal microscope.

Results and Discussions:

Following was the spectral data(IR and NMR) of compound **1**:

5-[(4H-1,2,4-triazol-4-yl)methyl]-2-[2-(2-chlorophenyl)-thiazolidin-4-one-1,3,4-oxadizole **(1)**. Yellowish white needles, yield: 64%; m.p. 110-113oC (EtOH); IR: 3234 (-CH, Ar),1546 (-C=N), 1674 (-C=O) cm-1; 1H NMR (CDCl3) ş 8.39 (s,1H, N-CH-S); 8.30 (s, 2H, triazole ring); 8.25 (s, 2H, NCH2);7.30-6.99 (m, 4H, phenyl); 3.60 (s, 2H, SCH2) reported elsewhere [14].

To elucidate whether the compound **1** inhibits the K-562, MCF-7, HCT-116 and MOlT-4 cell proliferation through induction of apoptosis, we examined the cell death in three cell lines by DNA fragmentation using PI staining. This is the quantitative technique used for apoptosis study. The apoptosis inducing potential of test compound against various cell lines is shown in the tables **1-4**.

Table 1: Apoptosis inducing potential of compound **1** in K-562 cell line:

Conc./Cell cycle	G_0-G_1	G_2-M	S	Apoptosis (SubG_0-G_1)
Control	39.85	2.27	57.88	11.04
50 µm	51.19	8.39	40.42	18.18
10 µm	41.09	6.20	52.72	26.85
1 µm	32.88	0.00	67.12	22.93
0.5 µm	36.62	2.84	60.54	22.62
0.25 µm	**47.07**	**8.50**	**44.42**	**45.97**

Table **1** shows that the proportion of cells in the sub-G1 region is increased from 11.04% in control untreated cell to 45.97% (at 0.25 µm concentration) in test compound treated cells at a time point of 48 h (**Fig.1)**. This result suggested that compound **1** is capable of inducing apoptosis in K-562 cells.

Table 2: Apoptosis inducing potential of compound **1** in MCF-7 cell line:

Conc./Cell cycle	G_0-G_1	G_2-M	S	Apoptosis (subG_0-G_1)
Control	59.34	9.73	30.93	0.24
50 µm	**59.90**	**9.44**	**30.66**	**0.46**
10µm	59.44	9.83	30.73	0.33
1 µm	59.12	9.23	31.65	0.32
0.5 µm	57.52	10.07	32.41	0.21
0.25 µm	58.52	8.57	32.91	0.16

Table **2** indicates that the proportion of cells in the sub-G1 region merely increased from 0.24% in control untreated cell to 0.46% (at 50 µm concentration) in test compound treated cells at a time point of 48 h (**Fig.1**). This result suggested that compound **1** was capable of inducing apoptosis in MCF-7 cells.

Table 3: Apoptosis inducing potential of compound **1** in HCT-116 cell line:

Conc./cell cycle	G_0-G_1	G_2-M	S	Apoptosis (sub G_0-G_1)
Control	63.90	13.39	22.71	1.72
50 µm	65.54	13.20	21.27	6.71
10 µm	65.74	13.28	20.98	3.96
5 µm	**67.96**	**13.26**	**18.79**	**6.79**
1 µm	58.25	14.01	27.74	4.04
0.5 µm	60.50	14.86	24.64	4.15
0.25 µm	64.49	13.28	22.22	4.96

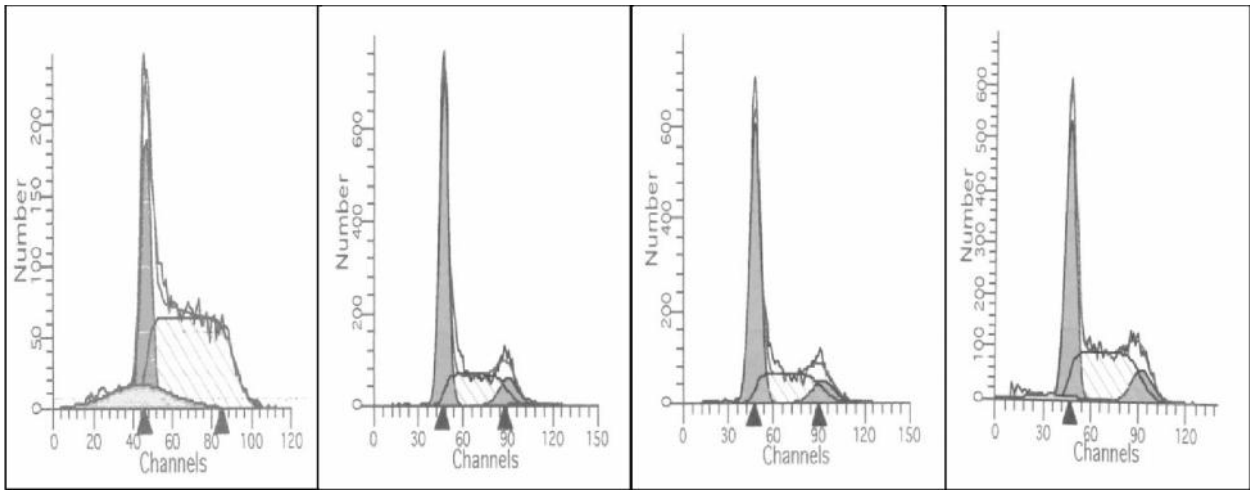
It is clear from the table **3** that the proportion of cells in the sub-G1 region increased from 1.72% in control untreated cell to 6.79 % (at 5 µm concentration) in test compound treated cells at a time point of 48 h (**Fig.1**). This result suggested that compound **1** was merely capable of inducing apoptosis in HCT-116 cells.

Table 4: Apoptosis inducing potential of compound **1** in MOLT-4 cell line:

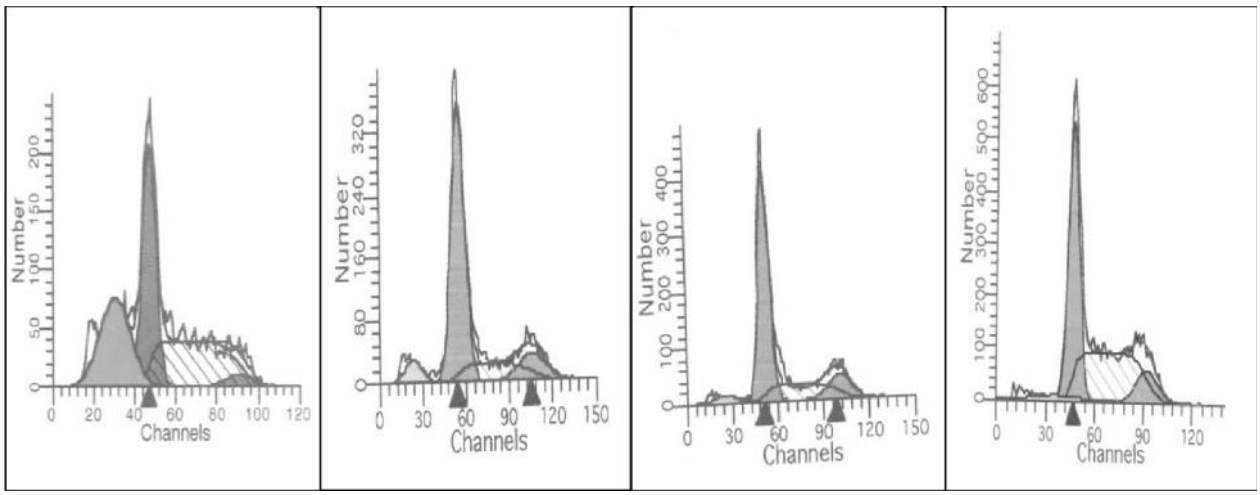
Conc./Cell cycle	G_0-G_1	G_2-M	S	Apoptosis (sub G_0-G_1)
Control	44.32	10.19	45.49	1.65
DMSO	43.94	9.81	46.26	2.23
50 µm	45.75	10.30	43.94	2.30
10 µm	44.73	11.06	44.21	2.03
1 µm	43.75	10.62	45.63	2.25
0.5 µm	45.73	9.60	44.67	2.04
0.25 µm	**41.99**	**8.54**	**49.47**	**3.46**

Table **4** suggests that the proportion of cells in the sub-G1 region increased from 1.65% in control untreated cell to 3.46% (at 0.25 µm concentration) in test compound treated cells at a time point of 48 h (**Fig.1)**. This result suggested that compound **1** was merely capable of inducing apoptosis in MOLT-4 cells.

K-562	MCF-7	HCT-116	MOLT-4



Control



Compound 1

Fig. 1 Apoptosis inducing potential of compound 1 in K-562, MCF-7, HCT-116, MOLT-4 cance r cell lines.

Table 5: Comparison between apoptosis inducing activity & *in vitro* anticancer activity (One Dose Assay) of compound **1**:

Activity/Cells	**K-562**	**MOLT-4**	**HCT-116**	**MCF-7**
Apoptosis* (%)	45.97	3.78	6.79	0.46
Anti-cancer activity #	8.59	20.20	13.29	8.09
(*Apoptosis Induction, # *In Vitro* anticance r activity in terms of % growth)				

Thus, compound **1** showed promising apoptosis inducing activity in K-562 cell line only, cell lines K-562 and MCF-7 exhibits good *in vitro* anticancer activity but the apoptosis inducing activity is very weak. Hence, it can be concluded that compound **1** shows anticancer action by induction of apoptosis in K-562 cell line only.

Laser Confocal microscopy was done on the cells (K-562 and MCF-7) treated with the compound **1** at different concentrations i.e. 0.25, 1 and 10 µM. Untreated cells were taken as negative control to compare the fragmentation with the treated cells.

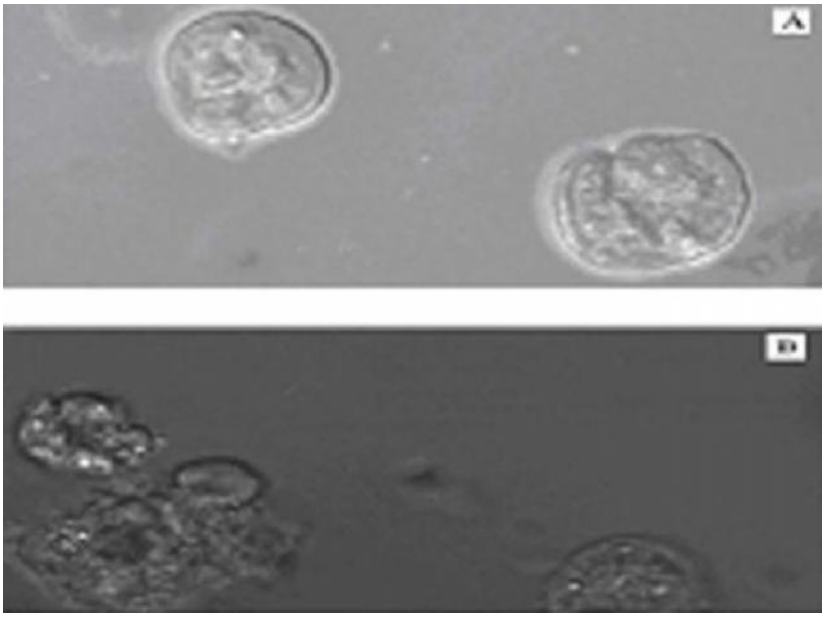


Fig.2 Laser confocal microscopic images in K-562 cell line (stained and non-stained): A. Untreated cells, Negative control, B. Compound 1 at 0.25 µm concentration.

In laser confocal microscopy **(**Fig.2), fragmentation of cells was observed when K-562 cells were treated with compound **1** at different concentrations. But, as expected, no fragmentation was observed in MCF-7 cells (data not shown). This observation is complimentary with the flow cytometric analysis. In that case also apoptosis induction was seen in K-562 cells only. Hence it can be concluded that the anticancer activity exhibited by compound **1** can be attributed to apoptosis induction in K-562 cells. But there could be another pathway/mechanism through which anticancer activity is exhibited by this compound in MCF-7 cells. This pathway needs to be thoroughly investigated and analyzed.

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