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The Pharma Innovation

ISSN (E): 2277-7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2023; 12(1): 767-771
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www.thepharmajournal.com
Received: 20-11-2022
Accepted: 24-12-2022

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Effect of cisplatin on daltons lymphoma ascites cancer cell lines

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Abstract
Cancer is one of the leading causes of death all over the world. A multifaceted approach is very much essential to treat the cancer patients along with the surgical interventions. One such approach is by using chemotherapeutic drugs. Cisplatin is one of the most commonly used chemotherapeutic drugs in use. Its exhibits its effects by binding to DNA and causing single stranded breaks. The present study was aimed to evaluate the anticancer property of Cisplatin in Daltons Lymphoma Ascites Cancer cell lines. Half-maximal inhibitory concentration (IC_{50}) of the drug was calculated as 10.76 µg/mL by performing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) Assay which revealed a significant concentration-dependent cytotoxicity. The mode of action of the drug was studied in detail by subjecting the Cisplatin treated cells to Acridine Orange / Ethidium Bromide (AO/EB) staining and assessing the relative expression of *Caspase-3* and *VEGF* genes. In AO/EB staining the cells treated with different concentrations of cisplatin emitted red florescence indicating apoptosis. The real time quantification revealed about 190-fold upregulation of *Caspase-3* and 14-fold downregulation of *VEGF* in cells treated with IC_{50} concentration of Cisplatin in comparison with control. These findings of the present study show that alternative effects like antiangiogenic effect along with activation of apoptotic pathway to be responsible of antineoplastic activity of Cisplatin.

Keywords: Cisplatin, daltons lymphoma ascites cancer cell lines, *caspase-3*, *VEGF*

Introduction

Cancer is one of the major causes of mortality in many countries and the second largest cause of death across the globe. There is a need of multifaceted therapeutic approach to address cancer in humans. This includes surgery along with chemotherapy, radiation therapy or immunotherapy depending on the stage of diagnosis (Bhat *et al.,* 2021) [2]. Chemotherapeutic drugs inhibit the growth of cancer cells by many ways. They act by blocking the production of DNA, mRNA or proteins through damaging DNA or by inhibiting the components that are essential for replication and chromosome separation (Petrović and Todorović, 2016) [13].

Cisplatin also known as Cis-Diamminedichloroplatinum (II) is a chemotherapeutic drug which is most commonly used to treat cancer. Cisplatin was first synthesised by Peyrone in 1845. Rosenberg and his colleagues generated Cisplatin accidently during the electrolysis of platinum electrode. The same team later studied the effect of Cisplatin in cancer cells. This led to a new era in cancer therapy. Cisplatin has a remarkable success in the effective treatment of ovarian, testicular, head and neck, oesophageal, cervical and lung cancers (Florea and Büsselberg, 2011) [6].

Caspase-3 has an essential role in apoptosis by cleaving various crucial cellular proteins. Devarajan *et al.* (2002) [5] reported a lack of caspase-3 activity in breast, ovarian, and cervical tumours. Cutone *et al.* (2020) [4] reviewed the biological mechanism of apoptosis, in which the protein binds to the TNF receptor 1 and Fas ligand (FASL) and forms a death-inducing signaling complex (DISC). The DISC activates the pro-caspase 8, which activates caspase-3 leading to the formation of the apoptosome, finally activating caspase-9 leading to apoptosis. Carmeliet (2005) [3] reported the VEGF as the critical mediator of angiogenesis in cancer. The upregulation of *VEGF* results in the formation of new vasculature in and around the tumour, resulting in the exponential growth of the tumour and this tumour vasculature is structurally and functionally abnormal.

With these backgrounds, the current study was designed to investigate the effect of Cisplatin in Dalton's Lymphoma Ascites (DLA) cancer cell lines.

Materials and Methods

Propagation of DLA cells

Dalton's lymphoma ascites cells were procured from Amala Cancer Research Centre, Thrissur. The cells were maintained continuously as ascitic fluid in the peritoneum by serial transplantation in stock group adult mice through intraperitoneal injection of 5×10^5 cells/mouse i.e., 2.5 million cells (counted in cell counter) (Thummar *et al.,* 2016) [18]. Cisplatin (Fresenius Kabi) which is available at a concentration of 1mg/mL was diluted with Phosphate Buffered Saline (PBS) to required concentrations.

Cytotoxicity studies using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) Assay

The cytotoxic changes produced by Cisplatin in DLA cells were assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) Assay. The cells were aspirated from the peritoneal cavity of tumour bearing mice, washed thrice with PBS and seeded at a density of 5×10^3 cells per well in 100µL medium. The cells were then treated with serially diluted Cisplatin @500 µg/mL, 250µg/mL, 125 µg/mL, 72.5 µg/mL, 36.25 µg/mL, 18.125 µg/mL, 9.06 µg/mL and 4.5 µg/mL) respectively for a period of 24 h. After the treatment, 10µL of MTT (5mg/mL) was added and incubated at 37ºC for four hours. The media with MTT was then removed and purple formazan crystals formed were dissolved in 200µL of Dimethyl sulfoxide (DMSO) and read at 570nm in ELISA plate reader (Varioskan Flash, Thermo Fisher Scientific, Finland). The percentage of cell inhibition was calculated which was then used to arrive at the inhibitory concentration 50 (IC_{50}) value by plotting it against the concentration using GraphPad prism version 5.

Acridine Orange / Ethidium Bromide (AO/EB) Staining

DLA cells were seeded into a 6- well plate at a concentration of 5×10^5 and treated with cisplatin for 24h. Cells of column A served as untreated control while those of columns B, C and D were treated with half the IC_{50}, IC_{50} and double the IC_{50} of Cisplatin. The Acridine Orange / Ethidium Bromide (AO/EB) staining procedure was followed to differentiate the live, apoptotic and necrotic cells. About 25 µL of the treated or untreated cells were stained with 5 µL of Acridine Orange (AO) (10 µg/mL) Ethidium Bromide (EB) (10 µg/mL) stain and analysed under Trinocular Research Fluorescence Microscope (Axio Vert. A1 FL-LED, Carl Zeiss, Jena, Germany) with blue excitation (488 nm) and emission (550 nm) filters at ×20 and ×40 magnifications (Ribble *et al.,* 2005) [16].

Relative gene expression studies

DLA cells were seeded into a 6- well plate at concentration of 5×10^5 and treated with $0.5 \times IC_{50}$, IC_{50} and $2 \times IC_{50}$ concentrations of Cisplatin for 24h. The cells were collected and centrifuged at 3000 rpm for five minutes. The pellet was washed with PBS (pH 7.4). Total RNA was isolated from the cells treated each concentration of the drug by TRIzol method (TriReagent, Sigma Aldrich, USA) followed by cDNA synthesis using verso cDNA synthesis kit (Thermo Fischer Scientific, USA). Optimum annealing temperature for the custom synthesized primers (Table 1) to amplify *Caspase-3* and *VEGF* genes as well as the internal control *GAPDH* gene was determined by gradient PCR. The expression of *Caspase-3, VEGF* and *GAPDH* was studied using SYBR green chemistry (Maxima SYBR green qPCR master mix (M/s Thermo Fischer Scientific, USA). The thermal cycler was pre-programmed for temperature and cycling conditions specified in Table 2. Melt curve analysis was performed and the relative expression of *Caspase-3* mRNA was calculated by $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) [10] by using *GAPDH* as reference gene. Post hoc test (Duncan's multiple range test) was conducted using SPSS V24.

Table 1: Details of primers for amplification of *GAPDH, CASPASE3*

Gene	Primer sequence	Product size (bp)
GAPDH	F:5′-AGGTCGGTGTGAACGGATTTG-3′	123bp
	R: 5′-TGTAGACCATGTAGTTGAGGTCA-3′	
VEGF	F: 5′-GCCAGCACATAGGAGAGATGAGC-3′	97bp
	R: 5′-CAAGGCTCACAGTGATTTTCTGG-3′	
CASPASE3	F: 5′-ATGGGAGCAAGTCAGTGGAC-3′	146bp
	R: 5′-GTCCACATCCGTACCAGAGC-3′	

Table 2: qRT-PCR conditions for *Caspase-3* and *GAPDH genes*

Steps			*Temperature*	*Time*
Initial denaturation			95 ºC	2 min
30 cycles of	Denaturation		94 ºC	1 min
	Annealing	*CASPASE3*	62 °C	30 sec
		GAPDH	60.8 °C	
		VEGF	65 °C	
	Extension		72 ºC	30 sec
Final extension			72 ºC	5 min

Results and Discussion

Cisplatin is a potent chemotherapeutic drug used effectively against various types of cancers including carcinomas, germ cell tumours, lymphomas and sarcomas in more than 50% of cancer patients. The present study evaluates the mode of action of Cisplatin in DLA cells lines.

The MTT assay was performed to investigate the cytotoxic potential on DLA cell lines. In normal live cells with active metabolism, MTT is converted to a purple-coloured formazan product with an absorbance near 570 nm. When there is cytotoxicity, decrease in the number of viable cells causes decreased purple formazan development where the change in absorbance can be measured by an ELISA plate reader (Mosmann, 1983) [20]. The percentage of cell viability and proliferation inhibition of DLA cells 24 h post-treatment with different concentrations of the anticancer drug Cisplatin are given in Table 3. Cisplatin displayed a significant effect on the viability of DLA cells in a dose dependent manner (Fig. 1) with a maximum reduction in percent cell viability at higher doses (500 µg/mL, 250 µg/mL, 125 µg/mL and 72.5 µg/mL). The IC_{50} value was obtained from the percent cell inhibition using Graph pad prism (Fig. 2) as 10.76 µg/mL. MTT assay gives a preliminary indication of the viability of cells and thereby the cytotoxicity of the compound under study.

Detailed mechanism of action of the agent has to be analysed using other assays.

Table 3: Percentage of cell viability of DLA cells, 24 h post treatment with various concentrations of Cisplatin

Conc. (µg/mL)	Cell Viability (%)	Cell Inhibition (%)
500	5.05±0.99 [a]	94.95±0.99 [a]
250	5.66±0.84 [a]	94.34±0.84 [a]
125	7.361±1.51 [a, b]	92.64±1.51 [a, b]
72.5	10.41±2.27 [a, b]	89.59±2.27 [a, b]
36.25	14.19±2.41 [b]	85.81±2.41 [b]
18.125	31.72±2.60 [c]	68.28±2.60 [c]
9.06	52.12±3.47 [d]	47.88±3.47 [d]
4.5	72.63±4.95 [e]	27.37±4.95 [e]

Values expressed as mean ±SE (n=6)

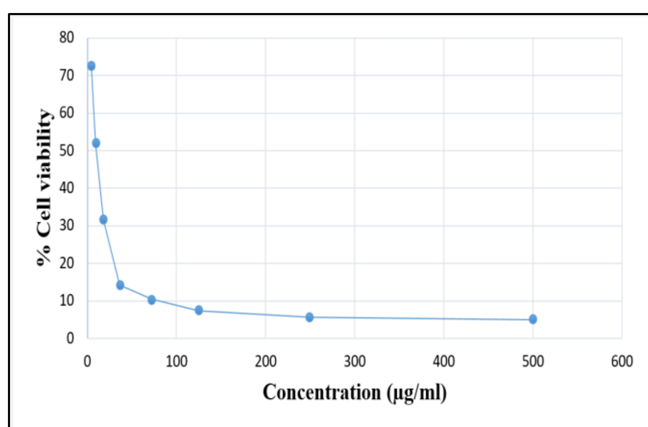


Fig 1: Percentage of cell viability of DLA cells, 24 h post treatment with various concentrations of Cisplatin

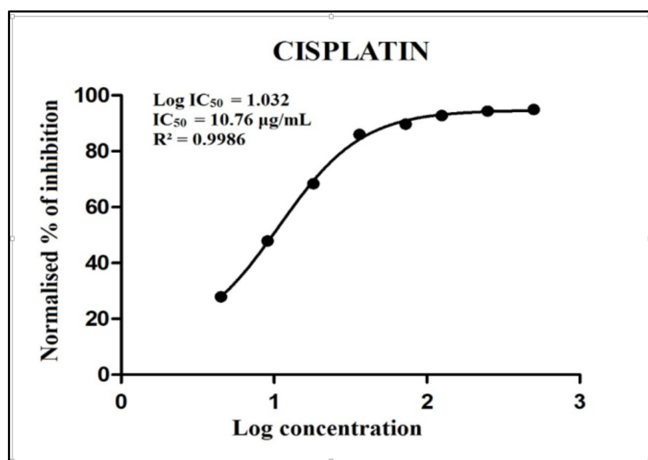
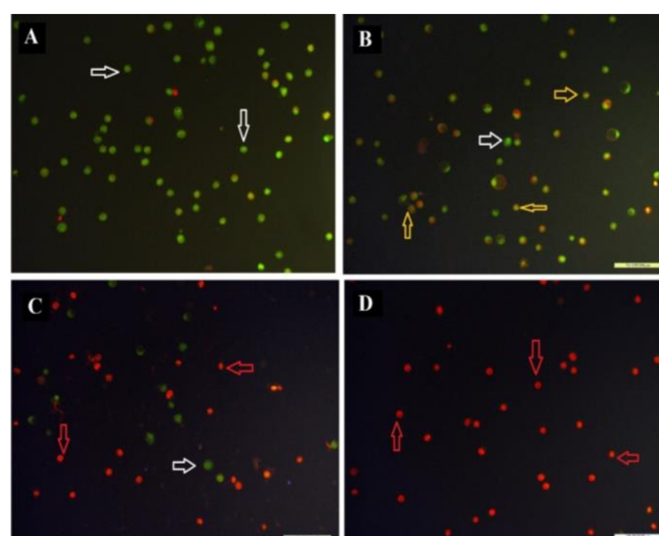


Fig 2: Dose-response curve of the percent inhibition of DLA cells against various log concentrations of cisplatin

The apoptotic changes in the Cisplatin treated cells were analysed by AO/EB staining (Fig. 3). This staining technique is used to identify the stage of both apoptotic and necrotic cells. AO stain diffuse into all the cells and emits green fluorescence upon binding to DNA. EB stain enters only the cells with compromised cell integrity like late apoptotic and necrotic cells. Early apoptotic cells show granular, yellow-green, crescent shaped marginalized nucleus. In late apoptosis, cells are either concentrated or shows asymmetric localized nucleus that are red in colour. Necrotic cells have increased cell volume with uneven orange red fluorescence at its periphery.

In the present study, the control cells emitted uniform green fluorescence with circular nucleus at the centre, whereas cells treated with Cisplatin @ 5 µg/mL (half IC_{50}) exhibited early apoptotic changes with orange-red fluorescence. Almost half of the cells treated with 10 µg/mL (IC_{50}) emitted red florescence indicating late apoptosis while the other half emitted green fluorescence. Almost the whole population of cells treated with double the IC_{50} concentration of Cisplatin @ 20 µg/mL emitted reddish fluorescence indicating late apoptosis. Cellular changes such as fragmentation, condensation, and marginalization of nucleus could be observed in the cells treated with Cisplatin. The integrity of cell membrane of late apoptotic cells is compromised because of which EB enters the cells and binds to concentrated DNA and apoptotic bodies producing orange to red fluorescence. Bendale *et al.* (2017) [1] and Ramezani *et al.* (2019) [15] also reported similar early-apoptotic, late-apoptotic, and some necrotic cells on treatment with cisplatin in cancer cells.



A- control, B- 5µg/ml of cisplatin, C- 10 µg/ml of cisplatin, D- 20 µg/ml of cisplatin, White arrow: live cells, Yellow arrow: early apoptosis, Red arrow: late apoptosis

Fig 3: Morphological changes of DLA cells treated with different concentration of cisplatin demonstrated by Acridine Orange / Ethidium Bromide (AO/EB) Staining.

Quantitative real-time PCR was conducted to analyze the relative tissue expression of *Caspase-3* and *VEGF* genes in Cisplatin treated DLA cells. A single peak in melt curve analysis of both the genes indicated the specificity of PCR reaction. The relative expression of *VEGF* and *Caspase-3* genes in the control cells was normalized to unity with *GAPDH* as the reference gene. In comparison to the control, the expression of *VEGF* gene was downregulated whereas the expression of *Caspase-3* gene was upregulated in the treated cells in a concentration dependent manner. The fold change in *VEGF* gene expression of IC_{50} and $2\times IC_{50}$ treatment groups was significantly ($p<0.05$) lesser than the control group while that of expression of *Caspase-3* was significantly ($p<0.05$) higher in all the treatment groups in comparison with the control and rcLf-N @ 0.5× IC_{50}. The maximum downregulation of expression of the angiogenic gene *VEGF* by about 111 folds and maximum upregulation in the expression of pro-apotoic gene *Caspase-3* by about 290 folds were observed in the cells treated with double the IC_{50} concentration (Fig. 4 and 5). The results show that Cisplatin

exerts its effects on DLA cells by bringing about apoptosis and by inhibiting angiogenesis. Similar results were reported by Ramer *et al.* (2018) [14], who reported the antiangiogenic effect of Cisplatin on lung cancer cells. In addition, Erlotinib-Cisplatin combination therapy on non–small cell lung cancer (NSCLC) *in vitro* was reported to down-regulate expression of *VEGF* (Lee and Wu, 2015) [8]. Liao *et al.* (2017) [9] reported synergistic effect of Cisplatin in combination with Matrine in inhibiting urothelial bladder cancer cells by down-regulating VEGF pathway, Zhong *et al.* (2007) [19] observed downregulation of VEGF in Cisplatin treated human ovarian cancer cells. Henkels and Turchi (1999) [7], Okamura *et al.* (2004) [12] and Sun *et al.* (2018) [17] observed similar findings and reported *Caspase-3*-dependent apoptosis in Cisplatin treated human ovarian cancer cell lines, Human hepatoma and oral squamous cell carcinoma cell lines and Non-Small Cell Lung Cancer respectively.

Table 4: Relative gene expression in DLA cells in response to treatment

Treatment groups	$\Delta C_T \pm$ SE		$\Delta\Delta C_T \pm$ SE		Fold change from control ($2^{-\Delta\Delta C_T}$)	
	VEGF	Caspase-3	VEGF	Caspase-3	VEGF	Caspase-3
Control	0.23±0.13	5.35±0.13	0±0.13	0±0.13	1 [a]	1 [a]
0.5xIC_{50}	1.35±0.74	0.30±1.31	1.12±0.74	-5.05±1.31	0.46 [a]	33.1 [b]
IC_{50}	4.05±0.55	-2.24±0.11	3.82±0.55	-7.59±0.11	0.07 [b]	192.3 [b]
2x IC_{50}	6.92±0.65	-2.83±1.3	6.69±0.65	-8.18±1.3	0.009 [c]	290 [b]

Values with different superscripts differs significantly ($p \leq 0.05$)

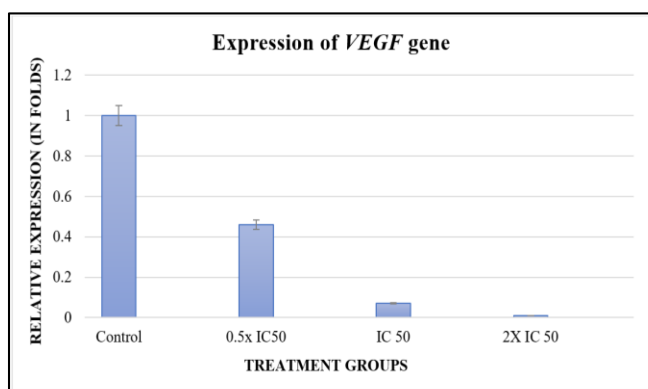


Fig 4: Relative *VEGF* gene expression in DLA cells in response to Cisplatin treatment

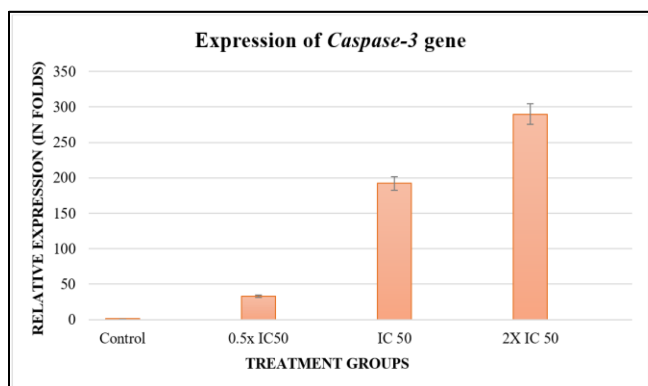


Fig 5: Relative *Caspase-3* gene expression in DLA cells in response to Cisplatin treatment

Conclusion

The effect of drugs or pharmaceutical agents could vary depending upon the type of cell they act on. The present study reveals that the anticancer mechanism of Cisplatin on DLA cancer cell lines could be contributed by several pathways such as induction of apoptosis and downregulation of angiogenic factors.

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