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Evaluation of the Efficacy of the Compound Actinomycin D in Tumor Cell Lines

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A B S T R A C T

The search for anticancer drug has lead researchers to study actinomycin D, a chromopeptide antibiotic that destroy cancer cells. The antitumor activity of actinomycin D analyzed on different cell lines by MTTassay. Different concentrations of compound (0.01-1.25µg/mL) was added to cell lines and incubated for 72 hrs. The compound actinomycin D showed cytotoxicity against U251, HCT-116 and MCF-7 cancer cell lines depending on the dose and incubation time. The best activity was shown against U251 cell line with IC_{50} value of1.07µg/ml on24 hr, 0.56µg/ml on48 hr and 0.028µg/ml on 72hr. The morphological change in actinomycin D treated U251 cells were detected by acridine orange and ethidium bromide staining. The comet assay revealed that actinomycin D inhibited the growth of U251 cells by induction of DNA damage.

Introduction

Actinomycin D is the best known compound from the actinomycin group whose wide application potential has been extensively studied. Actinomycin D is a orange red compound having two cyclic pentapeptide lactones attached to 2 amino-4,6-dimethyl-3-oxo-phenoxazine -1,9-dicarboxylic acid, first reported in *Streptomyces antibioticus* (Waksman and Woodruff, 1940). Subsequently number of other bacteria were also reported to produce actinomycin D including *Streptomyces fradiae* (Bossi *et al*., 1958), *Streptomyces parvulus* (*Williams* and Katz, 1977), *Streptomyces plicatus* (Lam *et al.*, 2002), *Streptomyces violascens* (Roos and Loane, 2004), *Streptomyces sindenensis* (Praveen *et al*., 2008) etc. Actinomycin D is found to be the most effective natural analog for the treatment of cancer in children and adults. It has been used clinically as a chemotherapeutic agent for the treatment of Wilm's tumor with 90% cure rate (Green, 1997; Farber *et al.*, 2002) and rhabdomyosarcoma (Womer, 1997). This compound was approved for the treatment of pediatric solid tumors by US FDA in 1964(Newman and Cragg, 2007). The biological activity is due to the abilityof actinomycin D to intercalate with the DNA double helix and there by inhibiting DNA directed RNA synthesis, this prevents unwinding of the DNA to facilitate its

interaction with RNA polymerase (Chou *et al*., 2002; Bendic, 2007).

The exciting potentialities of actinomycin D have attracted researchers to explore the medicinal value of actinomycin D.The present study was to investigate the cytotoxic potency against different tumor celllines and quantitative detection of DNA damage induced by compound.

Materials and Methods

Actinomycin D

The compound actinomycin D used in the present work was extracted from *Strptomyces parvulus* using methanol and purified by TLC following a standard protocol (Shetty, 2014). The compound was characterized using JEOL JMS 600 H mass spectrometer (JOEL, USA).

Cytotoxicity assay

The cytotoxicity activity of actinomycinD against human tumor cell lines HCT-116 (colon adenocarcinoma), MCF-7 (breast carcinoma) U251 (glioblastoma) was determined by MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) assay (Wilson, 2000). Briefly 1×10^4cells per well were seeded at log phase in 96 well plates. After cultivation for 24hrs the media was replaced with fresh media containing different concentration (0.01-1.25µg/ml) of actinomycin D dissolved in 0.1% DMSO and treated for 24, 48and 72 hr. After incubation at 37^0C in 5% CO2, 100µl of medium containing 20µl of MTT solution (5mg/ml in PBS) was added to each well and cells were incubated for 4hrs at 37^0C. At the end incubation, medium was removed and 100µl of DMSO was added to each well for formazan crystals to dissolve. The absorbance was measured at 570nm using Mullikan GO Micro plate Spectrophotometer (Thermoscientific). Doxorubicin was used for the positive control. The growth inhibition was calculated from the formulae (1-A/B)×100% where A and B corresponds to the mean absorbance of treated and control wells. The concentration of the compound that gives 50% inhibition was expressed as the IC_{50} (ug/ml).

Acridine orange and Ethidium bromide staining

To detect morphological changes induced by actinomycin D, briefly 1×10^4 U251 cells per well were seeded at log phase in 96 well plates and treated with 0.028µg/ml of actinomycin D for 72 hr. The untreated and treated cells were stained 20µl of of acridine orange and ethIdium bromide (100µg/ml each) and observed under a fluorescence microscopy (Leica, Germany).

Comet assay

Measurement of cellular DNA damage induced by compound was carried out using alkaline comet assay (Collins *et al*., 2008; Ramos *et al.*, 2010). Briefly, U251 cells were seeded in 24 well plates at a density of 1×10^5 cells per well for 24hr at 37^0C. After adhesion cells were treated with 0.028µg/mland 1.25 µg/ml of actinomycin D and incubated for 72h. After treatment, cells in each well were trypsined, centrifuged and resuspended in 1ml PBS. Samples were stored at 4^0C. A 10µL aliquot of resuspended cells were mixed with 70µl of 0.5% (w/v) low melting point agarose in PBS and transferred to 1% (w/v) agarose coated slides (normal melting point). Slides were immersed in lysis buffer (10mM Tris, 2.5M Nacl, 100mM EDTA, pH adjusted to 10 before addition of 1% Triton X-100) and kept at 4^0 C. After lysis the slides were

immersed in an alkaline electrophoresis buffer (300mM NaoH, 1Mm EDTA, pH 13) for 30 min at 4^0C. After a 20 min of electrophoresis by applying a current of 250mM, slides were removed and neutralized with two 5-min washes in a solution of 0.4 M Tris –HCl (pH 7.5), then fixed with 100% ethanol and dried at room temperature. After that slides were stained with ethedium bromide (20µg/ml) and examined under fluorescence microscope DNA migration was determined based on tail intensity (% DNA in tail) using a casplab software.

Result and Discussion

Cytotoxicity assay

To determine the cytotoxic activity of actinomycin D to tumor cell lines the cells were treated with increasing concentration of actinomycin D. The MTT cytotoxicity assay revealed that actinomycin D decreased the cell viability significantly($P<0.05$) in a concentration dependent manner. As shownin Fig.1 U251cells were more sensitive to actinomycin D compared to HCT and MCF7. Table 1shows the IC50 values of actinomycin D and doxorubicin against U251, HCT and MCF7.Actinomycin D showed a cytotoxic effect at an IC50 of 0.9µg/ml to U251 cells and to 1.09µg/ml to HCT-116 after 24hrs of treatment. However no IC50 was found on MCF7 at the same treatment duration. At 48hr the actinomycin D exhibited lowest IC50 Of 0.56µg/mltoU251 cells in contrast with the IC50 value of 1.03µg/ml to Hct-116 cells and IC50 value of 0.9µg/ml to MCF7cells. When the treatment was prolonged to 72 hr, actinomycin D exhibited IC50 value of 0.028µg/ml to U251, IC50 value of 0.55µg/ml to HCT-116 and 0.09µg/ml against MCF7. The The IC50 of purified actinomycin D comparable to standard doxorubicin.

There are reports of chemotherapeutic agents capable of inducing apoptosis, inhibiting cell proliferation or modulating signal transduction for the treatment of cancer (Bakshi *et al*., 2010). The results agree with previous investigation that actinomycin D has been shown to have cytotoxic activity against pancreatic cancer cell lines by inducing apoptosis (Kleeff *et al*., 2000).

Acridine orange and Ethidium bromide staining

The morphological changes suggestive of apoptosis were observed in U251 cells when treated with0.028µg/ml actinomycin D after 72 hrs was confirmed when cells were viewed after staining under fluorescence microscope Fig. 2.

Table.1 IC50values of actinomycin D and doxorubicin against different tumor cell lines

Compound	U251			Hct-116			MCF7		
	24hrs	48hrs	72hrs	24hrs	48hrs	72hrs	24hrs	48hrs	72hrs
Actinomycin D	0.9 µg/ml	0.56 µg/ml	0.028 µg/ml	1.09 µg/ml	1.03 µg/ml	0.55 µg/ml	-	0.9 µg/ml	0.09 µg/ml
Doxorubicin	-	1.15 µg/ml	0.5 µg/ml	0.9 µg/ml	0.48 µg/ml	0.18 µg/ml	1.20 µg/ml	1.1 µg/ml	0.84 µg/ml

Table.2 DNA damage induced by actinomycin D in U251 cell line

Parameter	Untreated	Treated(0.02µg/ml)	Treated (1.25µg/ml)
Tail length	6	17.33	26.93
Tail moment	0.01	2.11	11.65
% DNA in tail	99.79	87.59	44.594
OTM	0.1174	4.41	10.58

Fig. 1 Effect of actinomycin D (0.01 – 1.2µg/ml) on different tumor cell lines U251, HCT-116 and MCF7

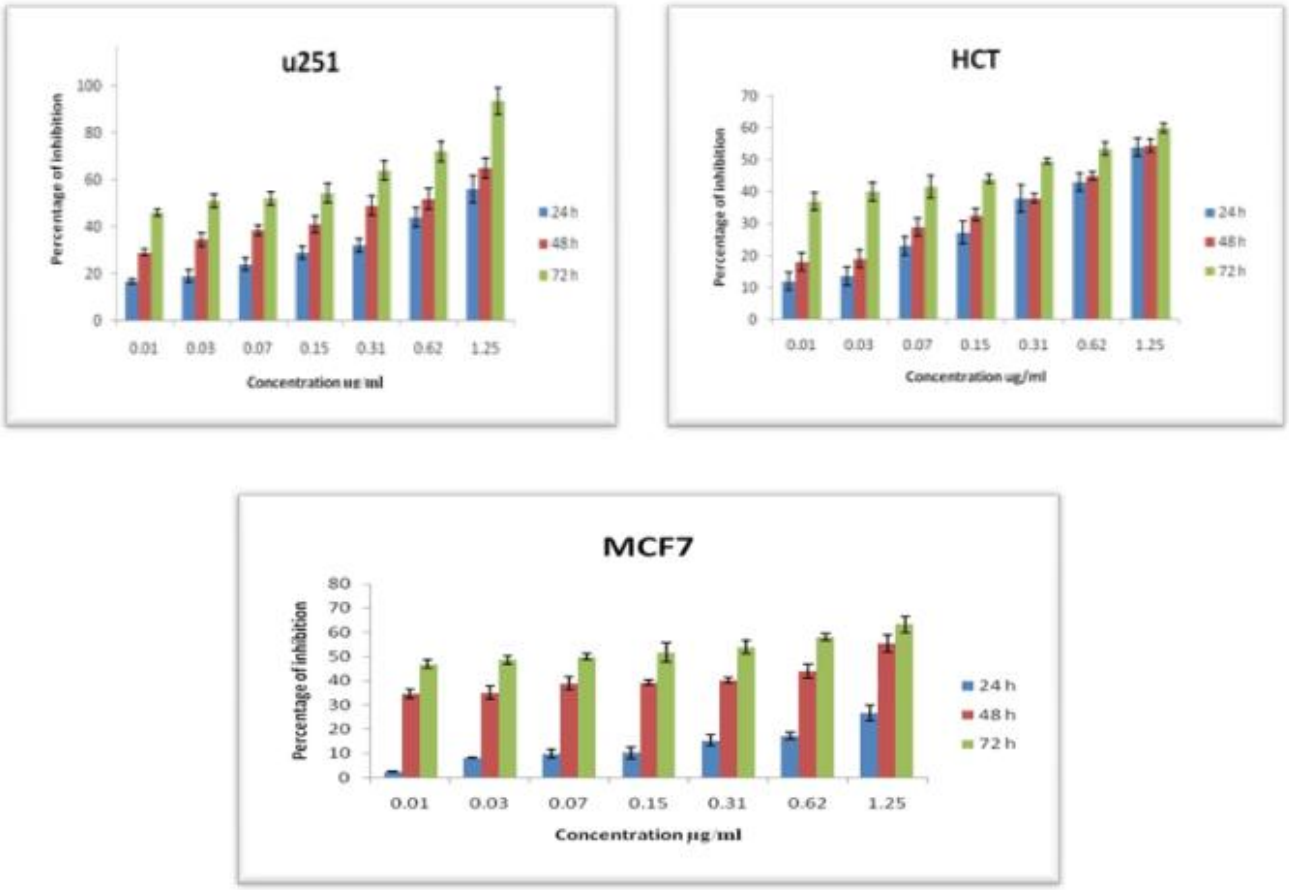
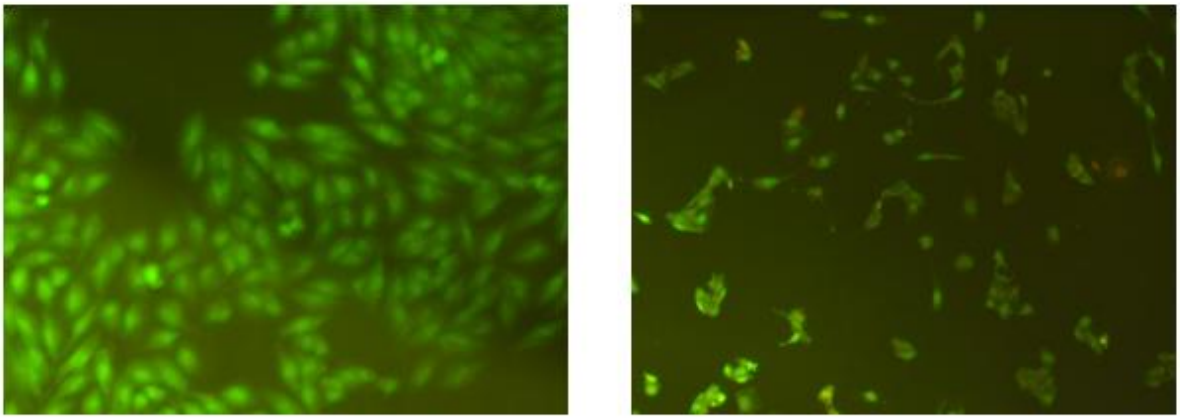


Fig. 2 Fluorescent microscopic images of actinomycin D treated U251 cell lines (a) control (b) treated



(a) (b)

The morphological changes induced by actinomycin D in U251 cells was further confirmed by acridine orange and ethidium bromide staining. There are reports of commercially available actinomycin D (0. 05µg/ml) inhibiting the growth of 60% glioblastoma cells after 92 hr by activating caspase-3 and 4 followed by apoptotic cell death (Narita *et al*., 2000). The concentration of compound increased the rate of inhibition in a dose dependant manner. Previous reports have shown that low doses of actinomycin D inhibit the cell proliferation of neuroblastoma cell lines by inducing apoptosis(Cortes *et al*., 2016).

Comet assay

Actinomycin D induce DNA strand breakage and formation of comets in U251 cells and in the untreated cells the nuclei were circular without formation of any visible tail as shown in Fig. 3. Percentage of cells with tail was significantly higher (P value ≤ 0.05) than the untreated cells. Tail lengths of the comets in cells treated with 0.028µg/ml was (17.33) and in 1.25µg/ml was found to be (26.93) and tail moment in 0.028µg/m treated cell was 2.11 and in cells treated with 1.25µg/ml found to be 11.65 shown in Table 2.

The level of DNA damage induced by compound to U251 cells was quantified by determining the intensity of DNA tail by comet assay(Tice *et al*., 2000, Hartmann *et al*., 2003)The present observation from comet assay indicates that cytotoxic effect induced by actinomycin D towards U251 may be caused by DNA damage.

In conclusion, the study has demonstrated for testing the cytotoxicity activity of actinomycin D against U251, Hct-116 and MCF-7cancer cell line. The U251 cells employed to assess the DNA damage induced by actinomycin D. The present study confirmed that actinomycin D induced potent cytotoxic activity and genotoxicity in U251 cells.

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