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Short communication

Inhibition of leukemic U937 cell growth by induction of apoptosis, cell cycle arrest and suppression of VEGF, MMP-2 and MMP-9 activities by cytotoxin protein NN-32 purified from Indian spectacled cobra (*Naja naja*) venom

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ABSTRACT

A cytotoxin NN-32 (6.7 kDa) from Indian cobra (*Naja naja*) venom inhibited human leukemic U937 cell growth as observed by Trypan blue dye exclusion method and cytotoxicity was confirmed by MTT assay. NN-32 induced apoptosis of U937 cell and cell cycle arrest of sub-G1 phase were revealed by FACS analysis. Increased Bax/Bcl-2 ratio, increased caspase 3 and 9 activities, cleaved PARP, decreased VEGF, MMP-2 and MMP-9 activities were observed after NN-32 treatment of U937 cell. Antileukemic activity of NN-32 on U937 cell may be due to activation of apoptosis, arresting cell cycle and antiangiogenesis activities.

Leukemia, a hematological malignancy caused by chromosomal aberrations or epigenetic alteration in hematopoietic stem cells (HSCs). It is characterized by uncontrolled multiplication of transformed blood cells specially leukocyte. Dysregulation of apoptosis, secretion of proangiogenic vascular endothelial growth factor (VEGF) are some of the characteristics of leukemia. Several treatment options present are still having side effects including emergence of drug resistance. Exploring natural products have become an approach for identification of novel clues for cancer therapy. Natural products derived compounds have been found to possess anticancer activity (Gordaliza, 2007). Snake venom being a natural product has enormous chemical diversity with potential biological activities. Snake venom cytotoxins are small protein with 60–62 amino acid residues (6.5–7 kDa) found abundantly in Elapid family of snakes. Several cytotoxins were identified from Indian spectacled cobra (*Naja naja*) which showed cytotoxicity on cancer cells (Hayashi et al., 1971; Takechi et al., 1972). A cytotoxin, NN-32 (6.7 kDa) was purified from *N. naja* venom by ion exchange chromatography using reverse phase HPLC using C4 column and showed its anticancer activity on Ehrlich ascites carcinoma in Balb/C mice model (Das et al., 2011). The present communication is an effort to establish antileukemic activity of NN-32 and its probable molecular mechanism of action on human leukemic cell line U937 (histocytic lymphoma).

NN-32 from *N. naja* venom was purified as stated earlier (Das et al., 2011). NN-32 caused dose and time dependent inhibition of U937 cell count as observed by Trypan blue dye exclusion method (Sur et al., 1995) shown in Table 1. NN-32

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Table 1
Effect of NN-32 on cell count and cytotoxicity of human leukemic U937 cell.

a. Effect of NN-32 on cell count of human leukemic U937 cell

Time (hrs)	NN-32 (μg/ml)			
	0.8	1.6	2.4	3.2
24	13.94 ± 0.7%↓	37.05 ± 2.4%↓	58.94 ± 2.4%↓**	66.94 ± 1.5%↓**
48	17.79 ± 2.2%↓	46.53 ± 1.0%↓	61.33 ± 1.7%↓**	79.81 ± 1.5%↓**
72	22.21 ± 1.1%↓	58.22 ± 0.8%↓	64.30 ± 0.9%↓**	87.63 ± 1.3%↓**

b. Effect of NN-32 on cell cytotoxicity of human leukemic U937 cell

Time (hrs)	NN-32 (μg/ml)		
	0.8	2.4	3.2
24	10.32 ± 0.5%↓	53.83 ± 1.5%↓**	64.44 ± 2.0%↓**
48	21.76 ± 3.9%↓	54.36 ± 1.7%↓**	76.58 ± 1.2%↓**
72	27.86 ± 1.6%↓	57.63 ± 1.6%↓**	79.56 ± 1.3%↓**

Values are % inhibition of U937 cell count and % reduction in O.D. at 570 nm (mean ± SEM, $n = 4$) (** indicates $p < 0.01$ significant). GraphPad software was used for statistics analysis and One- way ANOVA used for detection of difference between control and treated group followed by posttest analysis (significant level) by Dunnett's multiple test.

at dose of 0.8 μg/ml, 1.6 μg/ml, 2.4 μg/ml and 3.2 μg/ml at different time period showed significant inhibition of cell growth (Table 1a). IC50 value of NN-32 was found to be 2 μg/ml at 24 h. Cytotoxicity of NN-32 was confirmed by MTT (3-(4, 5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Cao and Li, 2002). NN-32 (at dose of 0.8 μg/ml, 2.4 μg/ml and 3.2 μg/ml) showed significant cytotoxicity toward U937 cell at different time period (Table 1b). Similarly, Cytotoxin NK-CT1 from Indian monocellate cobra (*Naja kaouthia*) venom showed U937 cell growth inhibition having IC50 value of 3.5 μg/ml (Debnath et al., 2010). Another basic protein Cardiotoxin III identified from *Naja atra* showed K562 (human leukemic cell line) cell growth inhibition with IC50 value 1.7 μg/ml (Yang et al., 2005).

There are several forms of cell deaths such as necrosis, apoptosis and autophagy (Kroemer et al., 2009). Cytotoxic agents exert their cytotoxicity via apoptosis or necrosis. To examine the effect of NN-32 on cell death, FACS was done to study annexin V-FITC binding. Data showed that there were 2.6% early ($PI^-/FITC^+$, LR quadrant), 23.6% late ($PI^+/FITC^+$, UR quadrant) apoptotic, and 61.03% viable ($PI^-/FITC^-$, LL quadrant) U937 cells after NN-32 (IC50) treatment, whereas control cell showed 0.14% cells in early apoptotic stage and 99.36% cells remained viable (Fig. 1A). These data indicated that NN-32 induced apoptosis on U937 cell. Apoptosis can be executed through mitochondrial and receptor mediated pathway. Intrinsic or mitochondrial mediated apoptosis may be triggered by cytotoxic drugs. Bcl-2 family proteins consisting of antiapoptotic as well as proapoptotic proteins play a key role in the intrinsic pathway of apoptosis. Antiapoptotic Bcl-2 protein, present in outer mitochondria membrane prevents apoptosis by preserving mitochondrial membrane integrity. Proapoptotic protein Bax, present in cytosol, moves into mitochondria during apoptosis and causes release of proapoptotic factors with subsequently activate caspase (Tzifi et al., 2012). After activation of initiator caspase, caspase 9 activates effector caspase, caspase 3 followed by DNA fragmentation, cleavage of damage repair enzyme, poly (ADP-ribose) polymerase or PARP. Western blot analysis indicated that NN-32 was able to downregulate Bcl-2 expression, upregulate Bax expression and cleave PARP in dose dependent manner in U937 cell (Fig. 1C). Caspase 3 and caspase 9 activities of cell lysate of U937 cells were measured using manufacturer's instruction. NN-32 (½ IC50 and IC50, 24 h) generated 1.3 fold and 2.2 fold increase in caspase 3 activities and 1.5 fold and 1.9 fold increase in caspase 9 activities respectively in U937 cells as compared to control. These data indicated that NN-32 might have a role in induction of apoptosis through mitochondrial pathway. Cytotoxin NK-CT1 from Indian *N. kaouthia* and Cardiotoxin III (CTX III) from *Naja naja atra* induced apoptosis by increasing Bax/Bcl-2 ratio, caspase 3 and caspase 9 activity in human leukemic U937and HL-60 cell (Debnath et al., 2010; Chien et al., 2008). However there are several similarities and dissimilarities observed between two cytotoxins.

The eukaryotic cell cycle is divided into four phases: G_1, S, G_2 and M. G_0 or sub-G_1 phase is defined as resting phase. Studies revealed that NN-32 (IC50) treated U937 cell showed 69.46% hypodiploid DNA in sub-G_1 phase, 14.21% DNA in G_1 phase, 3.01% DNA in S phase, whereas, control cells showed 4.14% DNA in sub-G_1 phase, 44.38% in G_1 phase, 12.94% in S phase (Fig. 1B). Due to increased amount of DNA accumulation in sub-G_1or G_0 phase after treatment it may be said that NN-32 arrested U937 cell at sub-G_1 phase or G_0 phase. Similar result was also observed after treatment with cytotoxin NK-CT1 from Indian *N. kaouthia* on leukemic cells (Debnath et al., 2010). Leukemic cells can be proliferated through the loss of cell cycle checkpoint controls. The cell cycle regulation is governed by cyclin dependent kinases (CDKs) consisting of kinase and cyclin. In order to assess the expression of proteins required for G_0 to S phase transition, western blot analysis was carried out. NN-32 downregulated CDK4 and cyclin D expression in cell lysate of NN-32 treated U937 cell as observed in western blot profile indicating role of CDK4, CDK6 and cyclin D in U937 treated cell cycle (Fig. 1C).

Various studies showed that angiogenesis played a pivotal role in leukemia (Reikvam et al., 2010). It was observed that VEGF (165) elicited survival of leukemic cells by induction of Bcl-2 expression and apoptotic inhibition (Dias et al., 2002). Recent studies showed that secretion of VEGF, matrix metalloproteinase MMP-2 and MMP-9 by leukemic cells increased the permeability of blood–brain barrier by disruption of endothelial tight junction protein