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Original Article

The anti-tumor efficacy of nanoparticulate form of ICD-85 versus free form

Zare Mirakabadi*[1], A., Moradhaseli[1], S., Sarzaem[1], A., Soheily[2], S., Morovati[3], H.

1. Department of Venomous Animal & Antivenom Production, Razi vaccine and Serum Research Institute, Karaj, Iran
2. Department of Biochemistry, Payam noor Tehran, Tehran, Iran
3. Department of Parasitology, Razi vaccine and Serum Research Institute, Karaj, Iran

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ABSTRACT

Biodegradable polymeric nanoparticles (NPs) have been intensively studied as a possible way to enhance anti-tumor efficacy while reducing side effects. ICD-85, derived from the venom of two separate species of venomous animals, has been shown to exhibit anti-cancer activity. In this report polymer based sodium alginate nanoparticles of ICD-85 was used to enhance its therapeutic effects and reduce its side effects. The inhibitory effect was evaluated by MTT assay. The necrotic effect was assessed using LDH assay. The induction of apoptosis was analyzed by caspase-8 colorimetric assay kit. Cytotoxicity assay in HeLa cells demonstrated enhanced efficacy of ICD-85 loaded NPs compared to the free ICD-85. The IC_{50} values obtained in HeLa cells after 48 h, for free ICD-85 and ICD-85 loaded NPs were 26±2.9µg ml^{-1} and 18±2.5µg ml^{-1}, respectively. While it was observed that free ICD-85 exhibits mild cytotoxicity towards normal MRC-5 cells (IC_{50}>60µg ml^{-1}), ICD-85 loaded NPs was found to have higher efficacy in anti-proliferative activity on HeLa cells in vitro without any significant cytotoxic effect on normal MRC-5 cells. The apoptosis-induction mechanism by both form of ICD-85 on HeLa cells was found to be through activation of caspase-8 with approximately 2 fold greater of ICD-85 loaded NPs as compared to free ICD-85. Our work reveals that although ICD-85 in free form is relatively selective to inhibit the growth of cancer cells via apoptosis as compared to normal cells, but nanoparticulate form increases its selectivity towards cancer cells.

Keywords: ICD-85, Sodium alginate, Nanoparticle, Necrotic effect, Apoptosis

INTRODUCTION

The efficacy of cancer chemotherapy is considerably limited by toxic side effects of anti-cancer drugs. This limitation can be due to the fact that conventional chemotherapy exposes both normal and cancerous cells (Na *et al* 2010). Hence, emphasis on optimizing the anti-tumor efficacy of cancer drugs while reducing the adverse effects on normal tissues is important issue. On the other hand, extensive efforts are also made on developing new therapeutic agents with improved safety and efficacy profiles (Hariparsad *et al* 2006). In order to enhance the potency of chemotherapeutic agents, new formulations to overcome the lacunas of conventional drug therapy is proposed (Peer *et al* 2007, Davis *et al* 2008). Nanotechnology is one such

*Author for correspondence.Email: A.zare@rvsri.ir

approach, where a constant dose of chemotherapeutic agents is delivered directly to the cancer cells over an extended period resulting in an alternative therapeutic approach for cancer therapy (Ferrari 2005, Seigneuric *et al* 2010). Nanoparticulate drug delivery system has many advantages like increased solubility and stability, normal tissues protection from toxicity, enhancement of pharmacological activities, improvement of tissue macrophage distribution and sustained delivery (Soppimath *et al* 2001, Nie *et al* 2007, Kumari *et al* 2010). Previous studies revealed that ICD-85 (venoms derived peptides) has inhibitory effect on the growth of various cancer cell lines including MDA-MB231 (Zare Mirakabadi *et al* 2008) and HL-60 (Zare Mirakabadi *et al* 2012). On the other hand DNA laddering and cell morphological studies confirmed that the inhibition of cancer cell lines by ICD-85 is through induction of apoptosis (Zare Mirakabadi *et al* 2012). In another *in vivo* study ICD-85 was able to prevent further growth of breast tumor and expand the life duration of mice with breast cancer (Koohi *et al* 2009). In this study, ICD-85 is selected as a cytotoxic agent and sodium alginate nanoparticles containing ICD-85 were prepared and evaluated their safeness and anti-cancer effects.

MATERIALS AND METHODS

Materials. The active fraction of ICD-85 is a combination of three peptides, ranging from 10,000 to 30,000 Da, derived from the venoms of snake (*Agkistrodon halys*) and scorpion (*Hemiscorpius lepturus*) was obtained from Razi Vaccine and Serum Research Institute (Karaj, Iran). The cell culture medium (DMEM), fetal bovine serum (FBS), trypsin-EDTA, penicillin and streptomycin were provided by Gibco (USA). 3-(4, 5-dimethyl-thiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), calcium chloride and dimethyl sulfoxide (DMSO) were purchased from Merck (Darmstadt, Germany). Sodium alginate and poly-L-lysine were purchased from Sigma-Aldrich Chemical (Germany). All other chemicals used in this study were of analytical grade.

Preparation of ICD-85 loaded NPs. The ICD-85 loading NPs were prepared by ionic gelation method as described in a previous report (Moradhaseli *et al* 2013).

Cell culture and proliferation. Human Cervix Carcinoma cell line (HeLa) and normal human lung fibroblast cells (MRC-5) were obtained from cell bank of Razi Vaccine and Serum Research Institute (Karaj, Iran). Cells were cultured in the DMEM medium, supplemented with FBS (10%), penicillin (100 Units ml^{-1}) and streptomycin (100µg ml^{-1}). The cells were grown at 37˚C with 90% humidity and 5% CO_2 (Freshney 2010). The cultured cells were treated with free ICD-85 and ICD-85 loaded NPs and examined their effects in different concentrations. The concentrations of ICD-85 loaded NPs treatments were calculated in such a way to include equivalent amounts as free ICD-85 treatments as previously described (Moradhaseli *et al* 2013). The cells were evaluated after 48 h of the treatment. Proliferation of human cancer and normal cells was performed by MTT assay as previously described (Moradhaseli *et al* 2013).

Lactate dehydrogenase (LDH) release assay. Membrane integrity was evaluated by measuring LDH released from cells into the culture medium for 48 h using a commercial LDH release kit (Roche Diagnostics GmbH, Germany). Experiments were performed as manufacturer's instructions. Absorbance values were determined photometrically with a 96-well plate reader (Bio-Tek, USA).

Morphological changes of cells. Free form of ICD-85 and sodium alginate encapsulated ICD-85 treated cells were observed under inverted microscope (Olympus CK2, Japan) and compared with untreated cells. Any change in morphological appearance of cells was considered in evaluation after 48 h post-treatment.

Caspase-8 assay. The activity of caspase-8 was examined using colorimetric assay Kit according to the manufacturer's instructions (BioVision, USA). Briefly, cells were treated with free ICD-85 and ICD-85 loaded NPs for 48 h. At the end of treatment, cells were pelleted by centrifugation and lysed on ice. Then, supernatants were collected and added into 96-well

plates (Nunc, Denmark). Final reaction buffer (50µl) and 5µl caspase-8 colorimetric substrate (IETD-pNA) were then added to each well. After incubation at 37˚C for 2 h, the release of pNA was measured at 405nm. The increase in the activity of caspase-8 was determined by comparing these results with the levels in untreated control.

Statistical analysis. Each experiment was repeated three times and the results were presented as mean ± SD. Means were compared using standard t-tests and the P-values are indicated in the figure legends. The half maximal inhibitory concentration (IC_{50}) values was determined using GraphPad Prism software. The results were considered statistically significant when P<0.05.

RESULTS

Cytotoxic effects on human cancer and normal cells. As the first step of our investigation, we assessed the effect of free ICD-85 and ICD-85 loaded NPs on the viability of HeLa cell line. Figure 1 shows the survival of HeLa cancer cells after 48 h of incubation with free ICD-85 and ICD-85 loaded NPs evaluated by MTT assay.

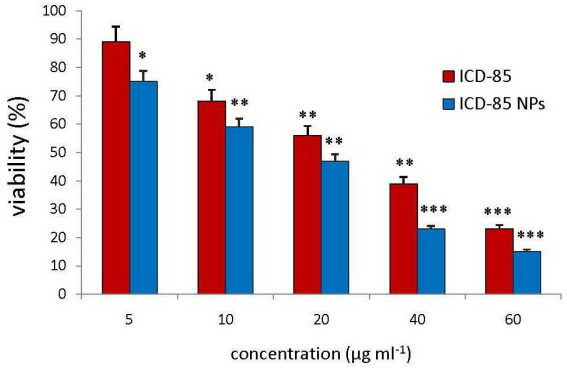

Figure 1. Viability of HeLa cells exposed to ICD-85 and ICD-85 loaded NPs at 48 h and measured by MTT assay. Viability of untreated cells (0µg) was taken as 100%. The measurements of the treated cells were normalized to the control measurement (100%). Results are expressed as mean ± SD of three or more independent experiments. *P<0.05, **P<0.01 and ***P<0.001 were considered to be statistically significant.

The results of the MTT assay showed a significant decrease in cell viability and increased number of non-viable HeLa cells as soon as 48 h exposure to free form of ICD-85 and ICD-85 loaded NPs, which occurred in a dose-dependent manner. The significant (P<0.001) growth inhibitory rate of ICD-85 loaded NPs at 60µg ml^{-1}on HeLa cells was 85%. Based on data collected from three independent experiments carried out in triplicate, the IC_{50} values obtained in HeLa cells for free ICD-85 and ICD-85 loaded NPs were 26±2.9µg ml^{-1} and 18±2.5µg ml^{-1}, respectively, after 48 h of incubation. In addition, we examined the effect of free ICD-85 and ICD-85 loaded NPs on the survival of normal MRC-5 cells (Figure 2). The comparison of the respective cell viability showed that normal cells are significantly (p<0.001) less sensitive to ICD-85 loaded NPs in vitro.

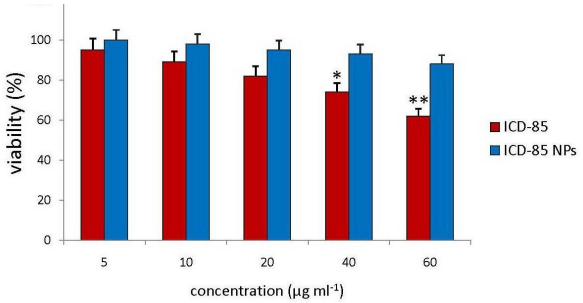

Figure 2. Viability of MRC-5 cells exposed to ICD-85 and ICD-85 loaded NPs at 48 h and measured by MTT assay. Viability of untreated cells (0µg) was taken as 100%. The measurements of the treated cells were normalized to the control measurement (100%). Results are expressed as mean ± SD of three or more independent experiments. *P<0.05 and **P<0.01 were considered to be statistically significant.

LDH release. Treatment of HeLa cells with free ICD-85 at concentrations of 5, 10 and 20µg ml^{-1} did not significantly increase LDH release but when free ICD-85 concentration increased to 40µg ml^{-1} and above, the LDH activity in the cultured media increased significantly (P<0.05). Moreover, LDH determination of cultured media of HeLa cells exposed to various concentrations of ICD-85 loaded NPs even at 60µg ml^{-1} revealed no significant difference between control and exposed cells (Figure 3). Treatment of MRC-5 cells with various concentrations of free ICD-85 revealed the significant increase in LDH activity at concentrations 20µg/ml and above. In contrast, the evaluation of the necrotic effect of ICD-85 loaded NPs showed no

significant difference between control and exposed cells (Figure 4).

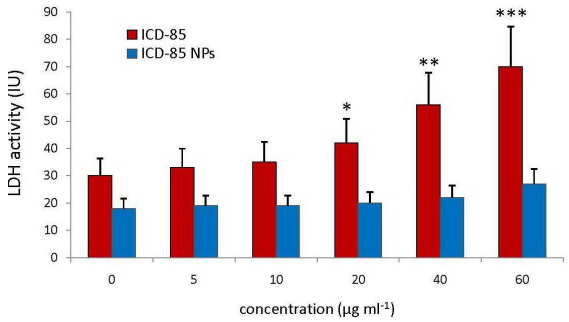

Figure 3. Activity of lactate dehydrogenase (LDH) after treatment of HeLa cells in the presence and absence of ICD-85 and ICD-85 loaded NPs for 48 h. Results are expressed as mean ± SD of three or more independent experiments. All the values are compared with values from control (0µg). *P<0.05, **P<0.01 and ***P<0.001 were considered to be statistically significant.

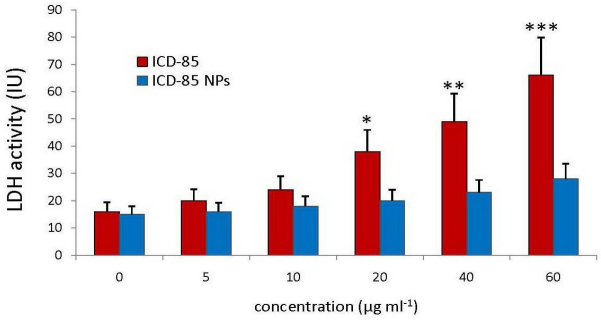

Figure 4. Activity of lactate dehydrogenase (LDH) after treatment of MRC-5 cells in the presence and absence of ICD-85 and ICD-85 loaded NPs for 48 h. Results are expressed as mean ± SD of three or more independent experiments. All the values are compared with values from control (0µg). *P<0.05, **P<0.01 and ***P<0.001 were considered to be statistically significant.

Morphological alterations. Morphological analysis showed significant difference between control cells (HeLa cells unexposed with free ICD-85) and treated HeLa cells with free ICD-85. The microscopic images of cells indicated that moderately cytoplasmic granulations of HeLa cells exposed to 60µg ml^{-1} of free ICD-85 occurred and a large number of cells became rounded in comparison with unexposed HeLa cell line (Figure 5b). The HeLa cells exposed to 60µg ml^{-1} ICD-85 loaded NPs exhibited obvious changes in morphologic characteristics. The HeLa cells were more rounding and granulation of cells in the ICD-85 loaded NPs treated group than in the control cells. The cell membrane was still preserved while the cytoplasm was full of vacuoles of different sizes (Figure 5c**).** Additionally, MRC-5 cells exposed to 60µg ml^{-1} of free ICD-85 revealed slight cytoplasmic granulations (Figure 5e). However, there was no significant morphological change observed in the ICD-85 loaded NPs treated MRC-5 cells at the same concentration (Figure 5f).

Caspase-8 activity. The effects of both form of ICD-85 on the activity of caspase-8 were investigated in HeLa and MRC-5 cells after 48 h of treatment. In HeLa cells, 20, 40 and 60µg ml^{-1} of free ICD-85 produced approximately about 1.7, 2.2 and 2.5 fold increase in caspase-8 activity (Figure 6). The assessment of caspase-8 activity showed a significant enhancement in HeLa cells with ICD-85 loaded NPs. HeLa cells treated with 20, 40 and 60µg ml^{-1} of ICD-85 NPs produced approximately about 2.9, 4.1 and 4.8 fold increases in caspase-8 activity respectively as compared to untreated control (Figure 6). ICD-85 loaded NPs treated HeLa cells showed approximately 2 fold more apoptosis compared to free ICD-85. MRC-5 cells exposed to free ICD-85 and ICD-85 loaded NPs revealed no significant increases in caspase-8 activity as compared to untreated control (data not shown).

DISCUSSION

Although conventional chemotherapies have traditionally been used to treat patients with various types of cancer, their side effects and damage to normal cells have been of monumental concern (Damon & Cadman 1988, Leszczyniecka *et al* 2001). The current studies were designed to investigate the safeness and anti-cancer effects of ICD-85 loaded NPs. Free form of ICD-85 was able to inhibit the growth of HeLa cells in a dose-dependent manner while it had mild effect on normal MRC-5 cell growth and viability at concentration of 60µg ml^{-1} which was 6-time higher than cytotoxic dose against HeLa cancer cells (10µg ml^{-1}). It seems that free ICD-85 had some selective anti-tumor effect. Treatment of HeLa cells with free form of ICD-85 resulted in the generation of apoptosis-

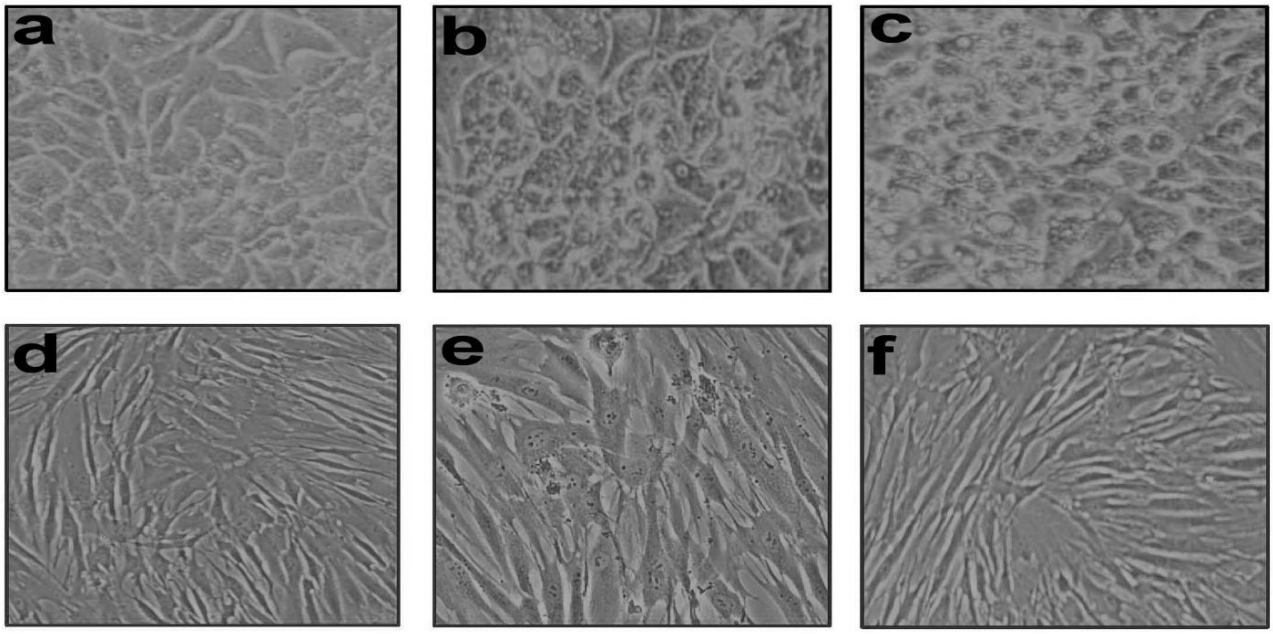

Figure 5. Morphological changes in the cells using inverted microscopy: (a) Untreated HeLa cells; (b) HeLa cells treated with 60µg ml^{-1} of free ICD-85 for 48 h; (c) HeLa cells treated with 60µg ml^{-1} of ICD-85 loaded NPs for 48 h; (d) Untreated MRC-5 cells; (e) MRC-5 cells treated with 60µg ml^{-1} of free ICD-85 for 48 h; (f) MRC-5 cells treated with 60µg ml^{-1} of ICD-85 loaded NPs for 48 hours.

specific morphological changes (Figure 5). The death receptor pathway, as the extrinsic apoptotic pathway, plays an important role in the induction of apoptosis (Gressner *et al* 2005, Kern *et al* 2006). We have observed that treatment with free form of ICD-85 resulted in the induction of apoptosis through activation of caspase-8 in HeLa cell line, suggesting that the inhibition of cell viability might be, at least in part, mediated by this mechanism. Moreover, as expected free form of ICD-85 does not significantly induce apoptosis in normal MRC-5 cells. Treatment of HeLa cells with free ICD-85 at concentrations up to 20µg ml^{-1} did not significantly increased LDH release, indicating that the treatment with free ICD-85 maintains the integrity of plasma membrane in HeLa cells. However, when concentration was above 40µg ml^{-1}, the LDH activity of free ICD-85 increased significantly ($P<0.05$). It is possible that increase in LDH activity of HeLa cells exposed to high concentrations of ICD-85 is due to necrotic effect rather than apoptotic effect which observed at low concentrations. An increase of LDH activity (as a marker of necrosis) in the culture supernatant indicated an increase in the number of dead or plasma membrane-damaged cells (Korzeniewski & Callewaert 1983). This suggests that particularly, in HeLa cells part of the apoptotic cells could switch to necrotic

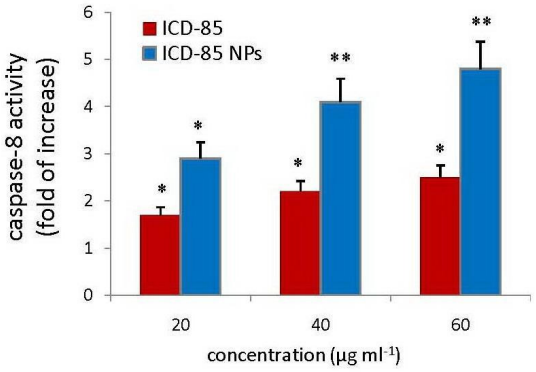

Figure 6. Caspase-8 activity in response to increasing concentrations of ICD-85 and ICD-85 loaded NPs (20, 40 and 60µg ml^{-1}) in HeLa cells following 48 h incubation. The activities were determined colorimetrically by using BioVision colorimetric caspase-8 assay kit. Results are expressed as fold increase in caspase-8 activity of control values ± SD of three independent experiments (**P*<0.05 and ***P*<0.01 relative to control).

mode of death after a prolonged time of incubation. Hence this part of present study showed the apoptotic nature versus necrotic effect of free form of ICD-85. To prepare ICD-85 loaded NPs we used sodium alginate. Alginate polymers have been widely used in biomedical applications as they are biodegradable, biocompatible and non-toxic (De & Robinson 2003, Rajaonarivony *et al* 1983). Moreover, previous studies in our laboratory had evaluated the potential cytotoxicity of sodium alginate NPs on HEp-2 cell line and we found that free sodium alginate NPs did not significantly affect the viability of HEp-2 cells (Moradhaseli *et al* 2013). These results indicated that free sodium alginate NPs itself did not affect the proliferation of cells. Therefore, we can anticipate that the interference of the carrier itself was negligible in this study. ICD-85 loaded NPs was able to inhibit the growth of HeLa cells in a dose-dependent manner. The IC_{50} values obtained in HeLa cells was reduced from 26±2.9µg ml^{-1} for free ICD-85 to 18±2.5µg ml^{-1}, for ICD-85 loaded NPs. These results showed that ICD-85 loaded NPs could significantly enhance the in vitro cytotoxicity against HeLa cells compared to the free ICD-85. Encapsulation of ICD-85 reduced cytotoxicity towards normal MRC-5 cells at least 3 fold as compared to free form of ICD-85. No rise in LDH activity was observed when the HeLa and MRC-5 cells treated with ICD-85 loaded NPs, in contrast to free ICD-85 which caused significant release of LDH at high concentration which suggests a response that caused by bursting of cell membranes during the process of cell necrosis. The absence of any appreciable LDH release detection accompanied with an anti-proliferative effect of ICD-85 loaded NPs could be an indication of the apoptosis. ICD-85 loaded NPs induced apoptosis to HeLa cells about 2 fold greater than free form of ICD-85 which is associated with the activation of caspase-8.

According to the results of the current study it is concluded that ICD-85 loaded NPs possesses higher efficacy in cancer cells inhibition while decreased toxicity towards normal cells.

Ethics

I hereby declare all ethical standards have been respected in preparation of the article.

Conflict of Interest

Hereby, I declare "no conflict of interest exists" regarding submitted article.

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References

Damon, L.E. and Cadman, E.C. (1988). The metabolic basis for combination chemotherapy. *Pharmacology & Therapeutics* 38: 73–127.

Davis, M.E., Chen, Z.G. and Shin, D.M. (2008). Nanoparticle therapeutics: an emerging treatment modality for cancer. *Nature Reviews Drug Discovery* 7: 771–782.

De, S. and Robinson, D.H. (2003). Polymer relationships during preparation of chitosan-alginate and poly-llysine-alginate nanospheres. *Journal of Controlled Release* 89(1): 101–112.

Ferrari, M. (2005). Cancer nanotechnology: opportunities and challenges. *Nature Reviews Cancer* 5: 161–171.

Freshney, R.I. (2010). Culture of animal cells: a manual of basic technique and specialized applications (6^{th} edn.). Pp: 187– 206. John Wiley & Sons, Inc., New York.

Gressner, O., Schilling, T., Lorenz, K., Schulze, S.E., Koch, A., Schulze-Bergkamen, H., Maria, L.A., Candi, E., Terrinoni, A., Valeria, C.M., Oren, M. and Melino, G. (2005). TAp63alpha induces apoptosis by activating signaling via death receptors and mitochondria. *The EMBO Journal* 24: 2458–2471.

Hariparsad, N., Sane, R.S., Strom, S.C. and Desa, P.B. (2006). In vitro methods in human drug biotransformation research: Implications for cancer chemotherapy. *Toxicology in Vitro,* 20: 135–153.

Kern, M.A., Haugg, A.M., Koch, A.F., Schilling, T., Breuhahn, K., Walczak, H., Fleischer, B., Trautwein, C., Michalski, C., Schulze-Bergkamen, H., Friess, H. and Stremmel, W. (2006). Cyclooxygenase-2 inhibition induces apoptosis signaling via death receptors and mitochondria in hepatocellular carcinoma. *Cancer Research* 66: 7059–7066.

Koohi, M.K., Zare Mirakabadi, A., Moharrami, M., Hablolvarid, M.H. (2009). Anticancer effect of ICD-85

(venom derived peptides) on MDA-MB231 cell line (in vitro) and experimental mice with breast cancer (in vivo). *International Journal of Veterinary Research* 3(1): 49–54.

Korzezeniewki, C. and Callewaert, D.M. (1983). An enzyme-release assay for natural cytotoxicity. *Journal of Immunological Methods* 64: 313–320.

Kumari, A., Yadav, S.K. and Yadav, S.C. (2010). Biodegradable polymeric nanoparticles based drug delivery systems. *Colloids and Surfaces B: Biointerfaces* 75(1): 1–18.

Leszczyniecka, M., Roberts, T., Dent, P., Grant, S. and Fisher, P.B. (2001). Differentiation therapy of human cancer: basic science and clinical applications. Pharmacology & Therapeutics *90: 105–156.*

Moradhaseli, S., Zare Mirakabadi, A., Sarzaeem, A., Mohammadpour dounighi, N., Soheily, S., and Rahimi Borumand, M. (2013). Preparation and characterization of sodium alginate nanoparticles containing ICD-85 (venom derived peptides). *International Journal of Innovation and Applied Studies* 4(3): 534–542.

Na, H.S., Lim, Y.K., Jeong, Y.I., Lee, H.S., Lim, Y.J., Kang, M.S., Cho, C.S. and Lee, H.C. (2010). Combination antitumor effects of micelle loaded anticancer drugs in a CT-26 murine colorectal carcinoma model. *International Journal of Pharmaceutics* 383: 192–200.

Nie, S., Xing, Y., Kim, G.J. and Simons, J.W. (2007). Nanotechnology Applications in Cancer. *Annual Review of Biomedical Engineering* 9: 257–288.

Peer, D., Karp, J.M., Hong, S., Farokhzad, O.C., Margalit, R. and Langer, R. (2007). Nanocarriers as an emerging platform for cancer therapy. *Nature Nanotechnology* 2: 751–760.

Rajaonarivony, M., Vauthier, C., Couarraze, G., Puisieux, F. and Couvreur, P. (1983). Development of a new drug carrier made from alginate. *Journal of Pharmaceutical Sciences* 82(9): 912–917.

Seigneuric, R., Markey, L., Nuyten, D.S., Dubernet, C., Evelo, C.T., Finot, E. and Garrido, C. (2010). From nanotechnology to nanomedicine: applications to cancer research. Current Molecular Medicine *10: 640–652.*

Soppimath, K.S, Aminabhavi, T.M., Kulkarni, A.R. and Rudzinski, W.E. 2001. Biodegradable polymeric nanoparticles as drug delivery devices. *Journal of Controlled Release* 70(1–2): 1–20.

Zare Mirakabadi, A., Mahdavi, S., Koohi, M.K. and Taghavian, M. (2008). Cytotoxic effect of ICD-85 (venom-derived peptides) on MDA-MB-231 cell line. *Journal of Venomous Animals and Toxins including Tropical Diseases* 14(4): 619–627.

Zare Mirakabadi, A., Shahramyar, Z., Morovvati, H., Lotfi, M. and Nouri, A. (2012). Induction of Apoptosis in Human Leukemia Cell line (HL60) by animal's venom derived peptides (ICD-85). *Iranian Journal of Pharmaceutical Research* 11(3): 931-938.