

Encapsulation in PLGA-PEG enhances 9- nitro-camptothecin cytotoxicity to human ovarian carcinoma cell line through apoptosis pathway

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Abstract

Ovarian cancer is the fifth leading cause of the cancer-related death among women. 9-nitrocamptothecin (9-NC) is a water-insoluble derivative of camptothecin used for the treatment of patients with advanced ovarian cancer. Previous studies showed that the encapsulation of 9-NC in poly (lactic-co-glycolic acid, PLGA) nanoparticles increased the cytotoxic effect of the drug on different cancer cell lines. In the present study, the cytotoxic effects of 9-NC, 9-NC-loaded PLGA and PLGA-polyethylene glycol (PLGA-PEG) nanoparticles with varying degree of PEG (5, 10, and 15%) were evaluated on human ovarian carcinoma cell line. Furthermore, the mode of cell death induced by 9-NC and the optimized 9-NC-loaded PLGA-PEG nanoparticles on A2780 cell line were investigated. 9-NC incorporating nanoparticles were prepared by nanoprecipitation method and their physicochemical characteristics were evaluated using standard methods. The results showed that activation of caspase-3 and -9 significantly increased by free 9-NC and PLGA-PEG loaded nanoparticles in A2780 cells. In contrast to the free drug which increased the activation of caspase-8, 9-NC-loaded PLGA-PEG nanoparticles did not alter the activation of caspase-8. Collectively, it appears that apoptosis induced by 9-NC incorporated in PLGA-PEG 5% occurred through the activation of caspase-9 rather than activation of caspase-8 which is the mediator of extrinsic pathway. Moreover, our results confirmed that 9-NC in nanoparticles at the level of gene expression potentiated down-regulation of Bcl-2, up regulation of Bax, and Smac/DIABLO leading to a decrease in mitochondrial membrane potential. Taken together, our results showed that 9-NC incorporated in PLGA-PEG 5% nanoparticles is able to induce apoptosis in A2780 human ovarian carcinoma cells and has the potential for the treatment of ovarian carcinoma.

Keywords: 9- Nitrocamptothecin; PLGA-PEG nanoparticles; Apoptosis; A2780 ovarian carcinoma cell line

INTRODUCTION

9-Nitrocamptothecin (9-NC) (Fig. 1) is one of the anticancer drugs that acting against a broad spectrum of cancers (1-3). Similar to the parent compound camptothecin, the derivative anticancer drug, 9-NC, interferes with the mechanism of action of the nuclear enzyme topoisomerase I (4). Previous studies have shown that the anti tumor activity of 9-NC is greater than parent drug camptothecin in human tumor xenografted in nude mice (4).

Unfortunately, at physiological pH, these potent agents undergo a rapid and reversible hydrolysis with the loss of antitumor activity (5-6). To overcome these problems, drug delivery systems such as liposomes, nanocapsules, micellar systems, and conjugates have been attempted (7). Previous researchers have shown that the encapsulation of the drug in poly (lactic-co-glycolic acid) (PLGA) nanoparticles increases its stability and its physicochemical characteristics such as solubility and release profile (8-10).

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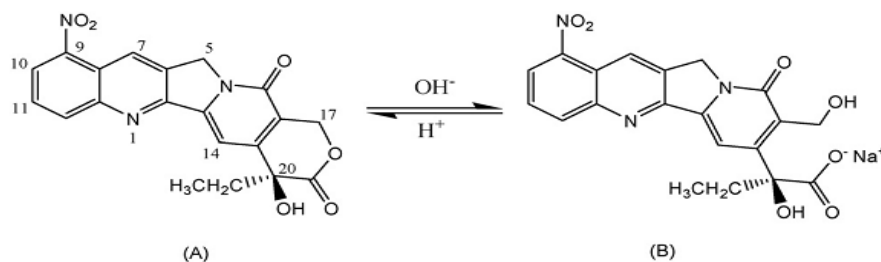


Fig. 1. The pH dependence equilibrium between lactone and carboxylate forms.

The apoptosis-induction capacity rather than necrosis induction is accepted as a key feature of a potential antitumor drug. Virtually all anticancer drugs induce apoptosis in susceptible cell types (11). This biochemically distinct form of cell death reflects, in large part, activation of caspases which are a distinct family of intracellular cysteine proteases (12). The caspases, has been shown to play a central role in initiation and execution of apoptosis. Upon activation, these enzymes cleave specific substrates and thereby mediate many of the typical biochemical and morphological changes in apoptotic cells (13).

Ovarian cancer is the most common cause of death among all women malignancies. Generally, the overall 5-year survival rate of patients with ovarian cancer is only about 30%, partly due to the absence of the symptoms at the early stages of the disease and poor prognosis (14). Activated apoptotic pathway by 9-NC per se in some clones derived from a human ovarian cancer cell line suggested that 9-NC alone may partially be effective for the treatment of patients with ovarian cancer (15). In our previous study, we demonstrated that exposure of A2780 human ovarian carcinoma cell line to 9-NC-loaded PLGA-polyethylene glycol (PLGA-PEG) nanoparticles increased the cytotoxicity of the drug (16). In the current study, we attempted to shed more light on the underlying molecular mechanisms through which the cytotoxicity of PLGA-PEG nanoparticles encapsulating 9-NC is mediated in A2780 human ovarian carcinoma cell line.

MATERIALS AND METHODS

Preparation of nanoparticles

PLGA and PLGA-PEG polymeric nanoparticles incorporating 9-NC were

prepared by nanoprecipitation technique. Briefly, the exact quantity of polymer and drug were dissolved in acetone and dispersed in the aqueous phase (pH, 3) containing polyvinyl alcohol as stabilizer.

The prepared nanoparticle suspension was stirred for 3 h at room temperature to evaporate the organic solvent. Subsequently, the nanoparticles were separated by ultracentrifugation (Beckman, XL-90, USA) at 30,000 rpm and 4 °C for 20 min and lyophilized using a lyophilizer (ZibusVaco 10-II-E; Germany) to obtain a fine powder of 9-NC-loaded nanoparticles (10).

The mean size and zeta potential of nanoparticles were measured by dynamic light scattering (DLS) (Zeta nanosizer, Malvern, France) at 25 °C and at a scattering angle of 90°. Encapsulation efficiency was determined by dissolving 20 mg of nanoparticles in 4 ml acetone by ultrasonicator (Tecna 3, USA). Concentrations of 9-NC in replicated samples were determined at 370 nm using a UV-Vis spectrophotometer (UVmini-1240, Japan). The encapsulation efficiency was calculated by the following equation:

$$\text{Encapsulation efficiency (\%)} = \left(\frac{\text{Drug quantity in nanoparticles}}{\text{Drug quantity originally used}} \right) \times 100 \quad (1)$$

Cell culture

A2780 cell line obtained from Pasteur Institute (Tehran, Iran) was incubated at 37 °C in a humidified atmosphere (90%) containing 5% CO₂ (Binder, Germany). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM-F12) (Ideh Zist, Iran) with 10% (v/v) fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin.

The medium was changed every two to three days and sub-cultured when the cell population density reached to 70–80% confluence.

Assessment of cell proliferation

The cytotoxic effects of free drug and drug-loaded nanoparticles were determined on A2780 cell line using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) (Sigma, USA) assay and compared with the untreated cells (17). 200 μ l of cells were plated onto 96-well plates at a density of 4.0×10^4 cells/well. Cells were then treated with various concentrations of 9-NC and 9-NC-loaded nanoparticles for 24 h. At appropriate time intervals, the medium was removed and replaced by 100 μ l of 0.5 mg/ml of MTT. Afterwards the plates were transferred to an incubator and kept for 3-4 h at 37 °C. Supernatants were removed and the reduced MTT dye was solubilized in 100 μ l dimethyl sulfoxide (DMSO) added to each well. Absorbances were measured on the enzyme-linked immunosorbent assay (ELISA) plate reader (Biotek, H1M.) at a test wavelength of 570 nm and a reference wavelength of 630 nm (OD570–OD630) to obtain sample signals. IC50 values were calculated by plotting the log10 of the proliferation percentage versus drug concentration.

Detection of caspase-3, -8 and -9 activation

Caspase-3, -8 and -9 assays were carried out using the sigma colorimetric caspase assay kit (Sigma, St Louis, MO, USA). This assay is based on the ability of the active enzyme to cleave the chromophore from the enzyme substrate, Ac-DEVD-pNA (for caspase-3), Ac-IETD-pNA (for caspase-8), and Ac-LEHD-pNA (for caspase-9) in equal amount of cells protein. The cells (5×10^6) were harvested and lysed in 70 μ l of the cell lysis buffer included with the kit, and protein concentrations were equalized for each condition. Subsequently, 10 μ l of cell lysate was combined with an equal amount of substrate reaction buffer containing caspase-3, -8 and -9 colorimetric substrates. This mixture was incubated for 2 h at 37 °C, and then absorbance was measured with a plate reader (BioTek, H1M,USA).

Analysis of apoptosis-related gene expression

To investigate the mitochondrial apoptotic events, changes in the levels of Bax, Smac/DIABLO, and Bcl-2 (β -Actin served as

intrinsic control) were determined. Total RNA was extracted from A2780 by high pure isolation kit (Roche, Mannheim Germany) according to the manufacture instructions. The primers used in this study were selected from published studies (18,19). Quality and quantity of total RNA were assessed using spectrophotometer (NanoDrop™ 2000, USA).

Thermal cycler conditions were 15 min at 50 °C to synthesize the cDNA, 10 min at 95 °C followed by 40 cycles of 15 s at 95 °C to denature the DNA and 45 s at 60 °C to anneal and extend the template. All reactions were performed in triplicate in a Corbett system (Australia). The value obtained for the target gene expression were normalized to β -actin and analyzed by the relative gene expression - $\Delta\Delta$ CT method where:

$$-\Delta\Delta CT = (CT_{\text{target}} - CT_{\beta\text{-actin}})_{\text{Unknown}} - (CT_{\text{target}} - CT_{\beta\text{-actin}})_{\text{calibrator}} \quad (2)$$

Measurement of mitochondrial membrane potential

Mitochondrial dysfunction has been shown to participate in the induction of apoptosis (20). In the present study, mitochondrial membrane potential (MMP) was measured using rhodamine 123 fluorescent dye. It is a cell permeable cationic dye that preferentially enters into mitochondria based on highly negative mitochondrial membrane potential (21). Depolarization of MMP (Ψ_m) during cell apoptosis results in the loss of rhodamine 123 from the mitochondria and a decrease in intracellular fluorescence intensity. After the treatment, A2780 cells were incubated with rhodamine 123 for 30 min at 37 °C. Then, cells were lysed with Triton X-100. The fluorescence was measured at an excitation wavelength of 488 nm and an emission wavelength of 510 nm using a fluorescence microplate reader (BioTek, H1M, USA).

Statistical analysis

Each experiment was performed at least three times, and the results were presented as mean \pm SEM. One-way analysis of variance (ANOVA) followed by Turkey's test was used to compare the differences between the means. A probability value of $P < 0.05$ was considered to be statistically significant.

Table 1. Formulations and characterization parameters of 9-NC nanoparticles.

Composition	External phase (ml)	Polymer (mg)	Emulsifier (mg)	Internal phase (ml)	Entrapment efficiency (%)	Particle size (nm)	PDI
PLGA	21.33	165	28	12	32	175	0.16
PLGA-PEG	18	125	50	4	45.7	134	0.23

Table 2. IC₅₀ Concentrations of free and loaded 9-NC in nanoparticles.

Group	IC ₅₀ ± SEM (μM)
Free drug	8.6 ± 0.23
PLGA	5.8 ± 0.35
PLGA-PEG 5%	1.01 ± 0.06
PLGA-PEG 10%	3.31 ± 0.51
PLGA-PEG 15%	1.89 ± 0.002

Data are expressed as the mean ± SEM of three separate experiments.

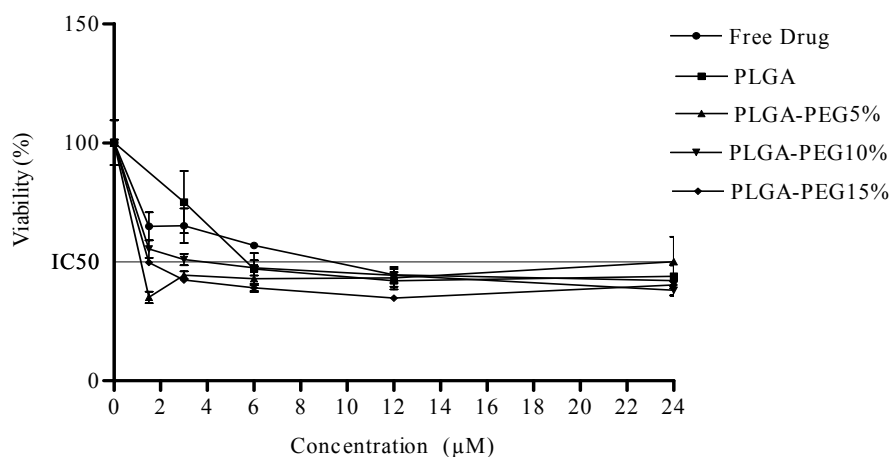


Fig. 2. Cytotoxic effects of 9-NC and 9-NC nanoparticles in A2780 cancer cells. The cells were incubated with different concentrations of 9-NC and 9-NC nanoparticles for 24 h. The cell proliferation inhibition was determined by MTT assay as described under materials and methods. Data are presented as mean ± S.E.M (n=3).

RESULTS

Nanoparticle preparation and characterization

The basic characteristics of the nanoparticles prepared in this study are presented in Table 1. The average diameter was 98 nm for PLGA nanoparticles and 112 nm for PEG-PLGA containing 5% PEG. Zeta potential for PLGA and PLGA-PEG nanoparticles were around 12.3 and 22.6 mV, respectively.

Determination of cytotoxicity

The concentration-response curves were generated and the drug sensitivity was expressed as a drug concentration that caused 50% growth inhibition (IC₅₀). As shown in

Fig. 2, treatment with 9-NC-loaded nanoparticles shifted the 9-NC concentration-response curve to a lower IC₅₀ values in a dose-dependent manner. The average IC₅₀ values of 9-NC, 9-NC-loaded nanoparticles are shown in the Table 2.

Based on these findings, PLGA-PEG 5% was able to transport more drug into the cells, thus achieving a lower cell viability and showed greater cytotoxicity compared to free drug or other nanoparticles after 24 h treatment. Therefore, PLGA-PEG 5% was selected for further experiments to elucidate the underlying mechanism of action of 9-NC loaded nanoparticles on A2780 cell line.

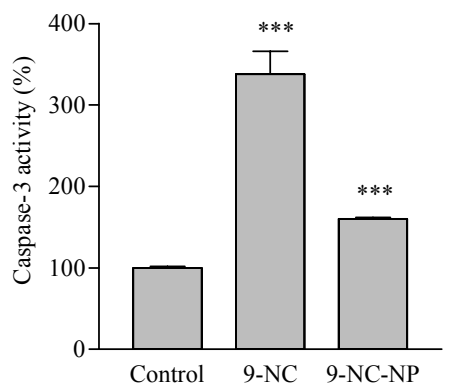
Determination of caspase-3, -8 and -9 activities

The findings showed that after exposure of the cells to the half maximal inhibitory concentrations (IC_{50}) of 9-NC and 9-NC-loaded PLGA-PEG 5% nanoparticles, caspase-3 as well as caspase-9 activations were increased significantly in A2780 cells compared to their respective controls (Figs. 3a and 3b). In contrast to the free drug which increased the activation of caspase-8, 9-NC-loaded PLGA-PEG nanoparticles did not alter the activation of caspase-8. Collectively, it appears that apoptosis induced by 9-NC incorporated in PLGA-PEG 5% occurred through the activation of caspase-9 rather than activation of caspase-8 which is the mediator of extrinsic pathway.

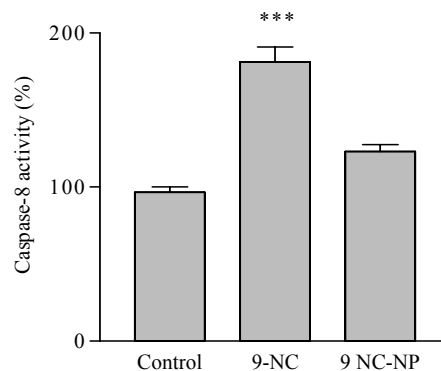
Determination of mRNA expression of some critical genes involved in apoptosis

Real-time RT-PCR analysis clearly showed

A)



C)



a significant reduction in the expression level of Bcl-2 after 24 h treatment with 9-NC-loaded PLGA-PEG 5% nanoparticles. Moreover, induction of apoptosis by 9-NC-PLGA-PEG 5% was accompanied by increase in mRNA expression of Bax and Smac/DIABLO genes. Surprisingly, we did not show any significant change in the mRNA expression of Bcl-2 and Smac/DIABLO genes upon treatment with 9-NC. Induction of apoptosis by 9-NC was only accompanied by increase in mRNA levels of proapoptotic Bax in A2780 cells (Fig. 4a-c).

Determination of MMP

We detected the effect of 9-NC and 9-NC-loaded PLGA-PEG 5% nanoparticles on the mitochondrial membrane potential in the A2780 cells. When A2780 cells were treated with 9-NC and 9-NC-loaded PLGA-PEG 5% nanoparticles for 24 h at 37 °C, a decrease in the retention of rhodamine 123 was observed (Fig. 5).

B)

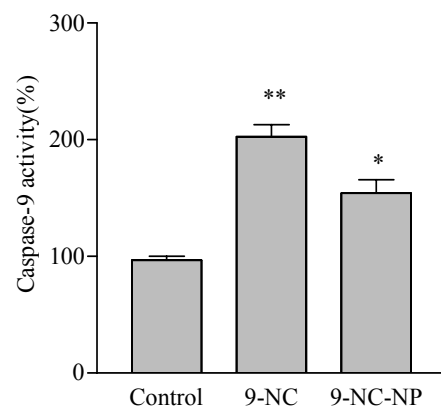


Fig. 3. Involvement of activation of A; caspase-3, B; caspase-9 and C; caspase-8 in the induction of apoptosis on A2780 human cancer cells. Cells were incubated with IC_{50} concentration of the 9-NC and 9-NC-PLGA-PEG 5% (9-NC-NP) and harvested at 24 h and cell lysates were assayed using microplate reader for activation caspases. Significant differences were compared with the control. Data are presented as mean \pm S.E.M. *; $P < 0.05$, **; $P < 0.01$ and ***; $P < 0.001$ vs. control.

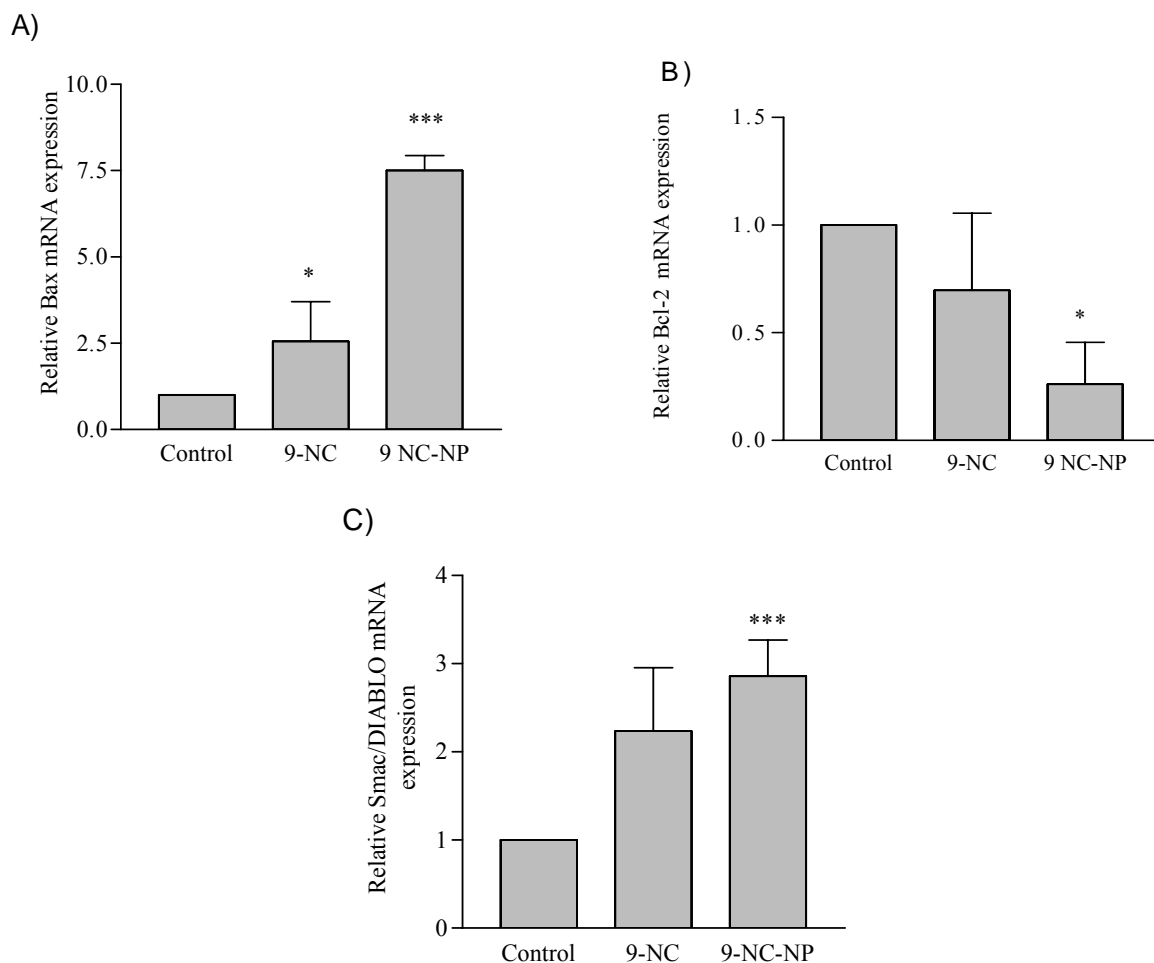


Fig. 4. The effect of 9-NC and 9 NC-PLGA-PEG 5% (9-NC-NP) on A; Bax, B; Bcl-2 and Smac/DIABLO mRNA expression and C; Bax/Bcl2 in A2780 human cancer cells. Normalization relative to b-actin was performed. Levels of mRNA are expressed relative to control in the mean \pm S.E.M values derived from three independent experiments.*; $P < 0.05$ and ***, $P < 0.001$ vs. control.

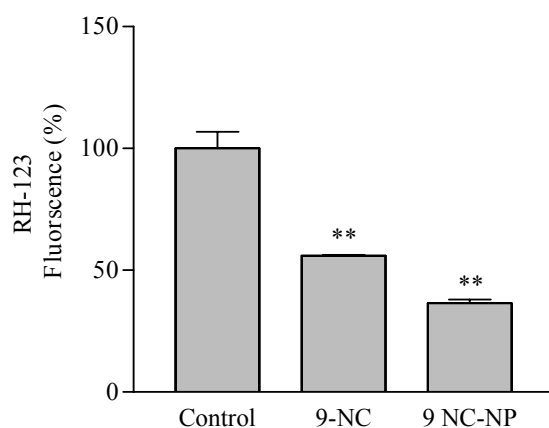


Fig. 5. The effect of 9-NC and 9 NC-PLGA-PEG 5% (9-NC-NP) on MMP in A2780 human cancer cells. Data are presented as mean \pm S.E.M, **, $P < 0.01$ vs. control.

DISCUSSION

This study was designed to evaluate the anticancer activities of 9-NC and 9-NC-loaded PLGA-PEG nanoparticles in human ovarian cancer cells. In the current study we concluded that 9-NC-loaded PLGA-PEG 5% is worthy of interest because of its high activity against A2780 cell line compared to other nanoparticles. This result is consistent to our previously described findings in which we showed that encapsulation of 9-NC in PLGA-PEG 5% nanoparticles could increase its cytotoxicity in A2780 cells (16). Since, the induction of apoptosis in tumor cells is considered very useful in the management and therapy as well as in the prevention of cancer, in the present study, we investigated the mode of cell death induced by 9-NC and 9-NC-loaded PLGA-PEG 5% nanoparticles by examining well-characterized apoptosis markers in A2780 cell line. The Bcl-2 family of proteins has emerged as a key regulatory component of cell death process, acting to either inhibit or promote cell death. Bax and Bcl-2, the two main member of this family influence the permeability of mitochondrial membrane (22). Permeabilization of mitochondrial membrane causes bioenergetics failure and permits the release of soluble molecules from the outer space of the mitochondria to the cytosol. One of the most important molecules called Smac/DIABLO cooperates in the cytosol to activate a cascade of caspases (22). Activation of the pro-apoptotic Bcl-2 family proteins triggers a cascade of signaling events in the mitochondrion-dependent apoptotic pathway ultimately leading to cytochrome c release and caspase-9 activation. Mature caspase-9 activates additional caspase-9 molecules as well as caspase-3 and caspase-7 and in turn the execution of cell apoptosis. In turn, caspase-3 activates downstream caspases in a proteolytic cascade (19,22). The results showed that activation of caspase-3 and -9 significantly increased by free 9-NC and PLGA-PEG loaded nanoparticles in A2780 cells. In contrast to the free drug which increased the activation of caspase-8, 9-NC-loaded PLGA-PEG nanoparticles did not alter the activation

of caspase-8. Collectively, it appears that apoptosis induced by 9-NC incorporated in PLGA-PEG 5% occurred through the activation of caspase-9 rather than activation of caspase-8 which is the mediator of extrinsic pathway. Similar results were also reported in the study by Chatterjee and coworkers, in which, 9-NC induced apoptosis through extrinsic pathway in DU145 human prostate carcinoma cells (23). In addition, it has been reported that the apoptotic pathway was activated by 9-NC in some clones derived from a human ovarian cancer cell (16). To gain a better understanding of how 9-NC and 9-NC-loaded PLGA-PEG 5% nanoparticles are able to induce apoptosis, we investigated apoptosis related genes expression. Our results confirmed that 9-NC-loaded PLGA-PEG 5% nanoparticles at the level of gene expression potentiated down regulation of Bcl-2, up regulation of Bax, and Smac/DIABLO leading to a decrease in mitochondrial membrane potential. Therefore, these effects could be attributed to an increased sensitivity of the A2780 cells to induced apoptosis when exposed to 9-NC-loaded PLGA-PEG 5% nanoparticles.

CONCLUSION

To summarize, 9-NC nanoparticles with 5% PEG-surface modification inhibited the growth of human ovarian carcinoma cells A2780 and induced mitochondria-related apoptosis. The more detailed mechanism of cell apoptosis by 9-NC-loaded PLGA-PEG 5% nanoparticles remains to be elucidated.

ACKNOWLEDGMENTS

The results presented here are extracted from the Pharm. D thesis of A. Jalalizadeh. This study was financially supported by the Research Council of Kermanshah University of Medical Sciences, Iran.

REFERENCES

1. Jiang Y, Sha X, Zhang W, Fang X. Complex of 9-nitro-camptothecin in hydroxypropyl- cyclodextrin: *In vitro* and in vivo evaluation. *Int J Pharm.* 2010;397:116-121.

2. Han X, Liu J, Liu M, Xie C, Zhan C, Gu B, *et al.* 9-NC-loaded folate-conjugated polymer micelles as tumor targeted drug delivery system: Preparation and evaluation *in vitro*. *Int J Pharm.* 2009;372:125-131.
3. Zheng Sh, Chang Sh, Lu J, Chen Zh, Xie L, Nie Y, *et al.* Characterization of 9-nitrocamptothecin liposomes: anticancer properties and mechanisms on hepatocellular carcinoma *in vitro* and *in vivo*. *PLoS One.* 2011;6:DOI:10.1371.
4. Giovanella BC, Hinz HR, Kozielski AJ, Stehlin JS Jr, Silber R, Potmesil M. Complete growth inhibition of human cancer xenografts in nude mice by treatment with 20-(S)-camptothecin. *Cancer Res.* 1991;51:3052–3055.
5. You J, Li X, Cui FD, Du YZ, Yuan H, Hu FQ. Folate-conjugated polymer micelles for active targeting to cancer cells: preparation, *in vitro* evaluation of targeting ability and cytotoxicity. *Nanotechnology.* 2008;19:102–111.
6. Sezgin Z, Yuksel N, Baykara T. Investigation of pluronic and PEG-PE micelles as carriers of meso-tetraphenyl porphine for oral administration. *Int J Pharm.* 2007;332:161–167.
7. Panaym J, Labhasetwar V. Biodegradable nanoparticles for drug and gene delivery to cells and tissue. *Adv Drug Deliv Rev.* 2003;55:329–347.
8. Derakhshandeh K, Erfan M, Dadashzadeh S. Encapsulation of 9-nitro camptothecin, a novel anticancer drug, in biodegradable nanoparticles: factorial design, characterization and release kinetics. *Eur J Pharm Biopharm.* 2007;66:34-41.
9. Derakhshandeh K, Soheili M, Dadashzadeh S, Saghiri R. Preparation and *in vitro* characterization of 9-nitrocamptothecin-loaded long circulating nanoparticles for delivery in cancer patients. *Int J Nanomedicine.* 2010;5:463-471.
10. Derakhshandeh K, Haghkhal M, Amiri M. Study of the copolymer structure effect on physicochemical characteristics and *in vitro* stability of PLGA-PEG nanoparticles loaded 9-nitrocamptothecin. *J Rep Pharm Sci.* 2012;1:107-117.
11. Réthy B, Zupk I, Minorics R, Hohmann J, Ocsovszki I, Falkay G. Investigation of cytotoxic activity on human cancer cell lines of arborinine and furanoacridones isolated from *Rutagraveolens*. *Planta Med.* 2007;73:41-48.
12. Hosseinzadeh L, Khorand A, Aliabadi A. Discovery of 2-Phenyl-N-(5-(trifluoromethyl)-1,3,4-thiadiazol-2-yl) acetamid derivatives as apoptosis inducer via caspase pathway with potential anticancer activity. *Arch Pharm (Weinheim).* 2013;346:812–818.
13. Czernski L, Nunez G. Apoptosome formation and Caspase activation: is it different in the heart? *J Mol Cell Cardiol.* 2004;37:643-652.
14. Baldwin LA, Huang B, Miller RW, Tucker T, Goodrich ST, Podzielinski I, *et al.* Ten-year relative survival for epithelial ovarian cancer. *Obstet Gynecol.* 2012;120:612-618.
15. Hu X, Balan KV, Ramos-DeSimone N, Wyche JH, Han Z, Pantazis P. Differential susceptibility to 9-nitrocamptothecin (9-NC)-induced apoptosis in clones derived from a human ovarian cancer cell line: possible implications in the treatment of ovarian cancer patients with 9-NC. *Anticancer Drugs.* 2003;14:427-436.
16. Dadashzadeh S, Derakhshandeh K, Shirazi FH. 9-nitrocamptothecin polymeric nanoparticles: cytotoxicity and pharmacokinetic studies of lactone and total forms of drug in rats. *Anticancer Drugs.* 2008;19:805-811.
17. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods.* 1983;65:55–63.
18. Mao HL., Liu PS., Zheng FZ, Zhang PH., Zhou LC, Xin G, *et al.* Transfection of Smac/DIABLO sensitizes drug-resistant tumor cells to TRAIL or paclitaxel-induced apoptosis *in vitro*. *Pharmacol Res.* 2007;56:483–492.
19. Porichi O, Nikolaidou ME, Apostolaki A, Tserkezoglou A, Arnogiannaki N, Kassanos A, *et al.* BCL-2, BAX and P53 expression profiles in endometrial carcinoma as studied by real-time PCR and immunohistochemistry. *Anticancer Res.* 2009;29:3977-3982.
20. Green DR, Reed JC. Mitochondria and apoptosis. *Science.* 1998;281:1309–1312.
21. Satoh T, Enokido Y, Aoshima H, Uchiyama Y, Hatanaka H. Changes in mitochondrial membrane potential during oxidative stress-induced apoptosis in PC12 cells. *J Neurosci Res.* 1997;50:413–420.
22. Hosseinzadeh L, Behravan J, Mosaffa F, Bahrami G, Bahrami A, Karimi G. Curcumin potentiates doxorubicin-induced apoptosis in H9c2 cardiac muscle cells through generation of reactive oxygen species. *Food Chem Toxicol.* 2011;49:1102-1109.
23. Chatterjee D, Schmitz I, Krueger A, Yeung K, Kirchhof S, Krammer PH, *et al.* Induction of apoptosis in 9-Nitrocamptothecin-treated DU145 human prostate carcinoma cells correlates with de novo synthesis of CD95 and CD95 ligand and down-regulation of c-FLIP (short). *Cancer Res.* 2001;61:7148–7154.