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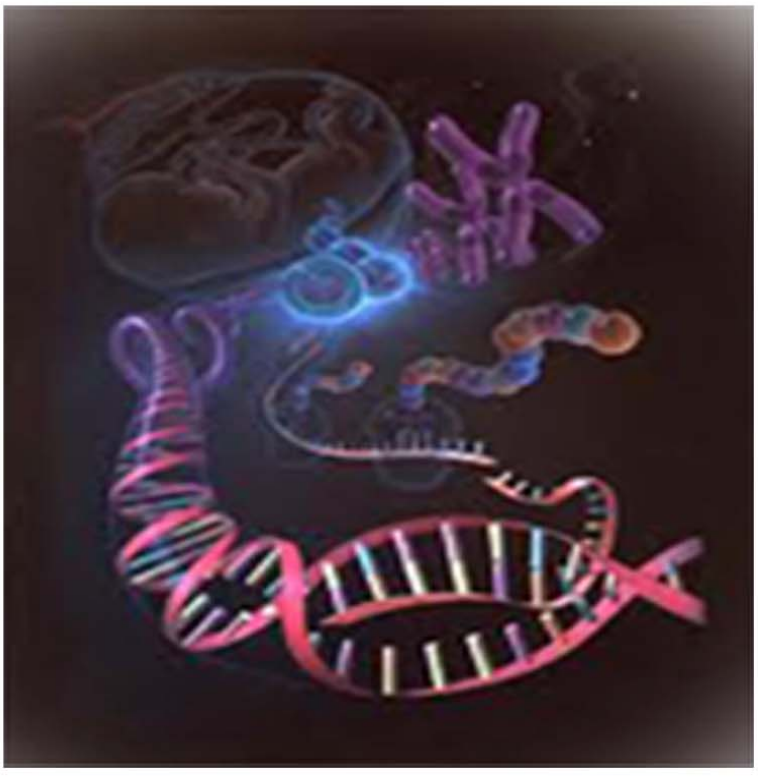

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Research Paper

EFFICACY OF NANOPARTICLE ENGINEERED DRUGS IN THE TREATMENT OF COLORECTAL CANCER AFTER FAILURE OF STANDARD CHEMOTHERAPY

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Chemotherapy represents a powerful therapeutic keystone in the development of present day cancer therapy. It is one of the most efficient and potent approach to treat colorectal cancer. On the other hand, the failure of chemotherapeutic drugs to selectively target cancer cells remains an important obstacle to successful chemotherapy. More or less all the anticancer drugs have severe side effects on normal tissues and organs. The undesired toxicity of currently available anticancer drugs and the inefficiency of chemotherapeutic treatments have limited the optimization of drug regimens and effective chemotherapeutic procedures. At present the field of nanomedicine allows the release of anticancer drugs by biodegradation and self-regulation of nano materials in vitro and in vivo. For that reason, a need is felt to entrap these drugs into enhanced drug delivery carriers such as SLN to minimize systemic side effects. In the current study SLN was prepared by solvent injection method and built-in with 5-fluorouracil, irinotecan and Ca-leucovorin. In this study we evaluated the apoptotic potential of the nano-engineered formulations was investigated using cultured HT-29 cells. Evaluation of anti-carcinogenic potential by Annexin-V-FITC/PI apoptosis assay and caspase-3 following dose dependent experiments treatment with SLN and native drugs described significant differences, creating better prospective efficacy of nano-engineered drugs. The result indicates that the SLN is a promising controlled release carrier. Nanoparticles as drug delivery systems allow exclusive approaches for cancer treatment.

Keywords: Colorectal cancer, Chemotherapy, Toxicity, Nanoparticles, Cytotoxicity, Apoptosis

INTRODUCTION

Colorectal cancer (CRC) is the third most widespread cancer and the fourth most frequent cause of cancer deaths worldwide (Haggar and Boushey, 2009). Among all cases of CRC, the majorities (~ 75%) are sporadic in origin and the remaining is related to inflammatory bowel diseases or family history (Hisamuddin and Yang, 2004).

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Conventional chemotherapy utilizes medications that are known to kill cancer cells successfully. In a metastatic site, patients are treated with standard chemotherapeutic regimens broadly used in colorectal cancer is composed of FOLFIRI, consisting of 5-fluorouracil (5-FU; blocks thymidylic acid formation with a well known inhibitor of thymidylate synthetase) with leucovorin (LV; a folate that helps in enhancing 5-FU to thymidylate synthase) and irinotecan (a powerful inhibitor of topoisomerase inhibitor that ultimately leads to inhibition of both DNA replication and transcription) (Roh *et al.*, 2012). But these cytotoxic drugs harness the host's immune system in addition to tumor cells, leading to severe ill effects such as nausea, neuropathy, hair-loss, tiredness, and compromised immune function (Rani *et al.*, 2012).

In present years, the progress of new drug alone is not enough to offer the basis for the development in drug therapy. A promising approach to conquer these inconveniences involves the need to unravel new drug carrier system.

Nanotechnology is a multidisciplinary field that put forward the means to intend therapies directly and specifically at cancerous cells. Nanoparticles are with their unique features based in intrinsic properties such as small particle size, large surface area and the potential of changing their surface properties have several advantages compared with other delivery systems (Kreuter, 2001). Nanoparticles are biodegradable and non-toxic solid colloidal particles ranging from 10 to 1000 nm (1.0 μm), in which the drug are suspended, entrapped, and/or to which the drug is adsorbed or attached (Chowdary *et al*., 1997). Nanoparticles can be used as drug carriers for chemotherapeutics to carry drug directly to the tumor while sparing healthy cells. Nanoparticles can also be engineered to deliver multiple drugs that are carried together in one particle with controlled release of each drug, preventing the requirement for complex multi-drug dosing regimens and improving patient compliance (Alexis *et al*., 2010).

Solid lipid nanoparticles are submicron sized colloidal carrier, which consists of physiologically biodegradable lipids. SLNs first introduced by Muller (Muller *et al.,* 1995). Which are used in the manufacturing of nanoparticles, are solid at room temperature and also at body temperature and with mean diameter approximately between 50 and 100 nm. Solid lipid nanoparticles have several advantages over conventional chemotherapy such as better physical stability, protection of incorporated drugs from being degraded in the body before they reach their target cell, increase the absorption of drugs into tumors and into the cancerous cells themselves, controlled release and minimizing the related problem (Garud *et al.,* 2012). For high drug loading and entrapment efficiency, lipophilic drug with good compatibility with lipids, have often been selected to incorporate in to SLN.

The purpose of this in vitro study was to investigate the efficacy of SLN entrapped drug over conventional regimens was detected by flow cytometry. To measure apoptosis, the Annexin-V-FITC/PI assay kit from Roche Applied Sciences, Mannheim, Germany was used. The activity of caspase-3 was quantified by using PE Active Caspase-3 Apoptosis kit from BD™ Biosciences, San Diego, CA, USA.

MATERIALS AND METHODS

The Human Colorectal adenocarcinoma, HT-29 cell line was obtained from NCCS, Pune, India. The cells were seeded at 2 x 10^5 cells/60 mm culture dishes in DMEM supplemented with 10% fetal calf serum plus 1.5 g/L sodium bicarbonate. Cultures were incubated the humidified atmosphere of 5% CO_2 at 37°C according to NCCS catalogue instructions. After optimum confluency, the cells were treated with the experimental agent, free drugs and encapsulated drugs and harvested with trypsin-EDTA for use in the following experiments.

Study Design

Dose dependent response of native and nano-engineered drugs on colorectal cell line was carried out with concentration ranging from 0.1 to 100 μg.

Detection of Apoptosis by Flow Cytometry

The apoptotic effects of the particular drug regimens were studied using an Annexin V and Propidium Iodide (PI) double-labelling procedure. In the early stages of apoptosis, the cell membrane remains intact and is impermeable to the DNA binding dye, PI. At this same stage, the phosphatidyl serine residue to which Annexin V specifically binds is translocated to the extra cellular leaflet of the membrane, making it accessible to Annexin V. In contrast, during necrosis, cells take up propadium iodide because the cell membrane is ruptured. Thus, cells which take up both fluorochromes are a combination of apoptotic and necrotic cells, whereas cells that exclude propadium iodide but bind Annexin V are (early) apoptotic cells. Measurement of apoptotic index of cultured cells was performed using Annexin-V-FITC/PI assay kit as per manufacturer's recommendations. From each cell, Forward light scatter (FSC), orthogonal light scatter (SSC), and Annexin-V-FITC and PI fluorescence were measured using Cell-Quest Software (BD-IS, USA). The gate was applied in the FSC/SSC dot plot to restrict the analysis to cultured cells only. For the gated cells, the percentages of annexin-V-FITC positive or negative or PI positive or negative cells were evaluated. In each case total 10,000 events were recorded in HI mode with 10/10 log quadrant gate.

Caspase-3 Activity

Caspase-3 is responsible for the cleavage of key cellular proteins, such as cytoskeletal proteins, that leads to the typical morphological changes observed in cells undergoing apoptosis. As such, it is a critical executer of apoptosis. The activity of active caspase-3 was measured by washing the cells with cold 1 X PBS and then resuspending in BD cytofix/cytoperm solution at a concentration of 1 X 10^6 cells/ mL followed by incubation of 20 min. on ice. The cells were then harvested and washed followed by incubation with antibody for 30 min. at room temperature. The cells were washed and analyzed by flow cytometry in FL2 channel.

RESULTS AND DISCUSSION

Annexin V

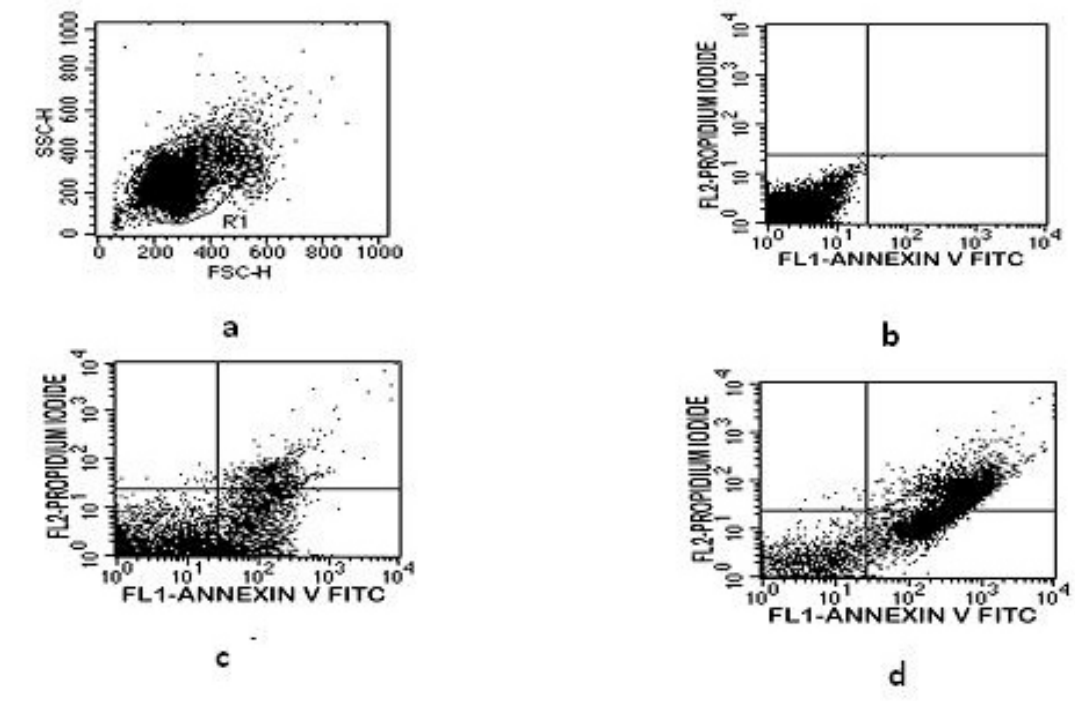
Annexin V is a Ca^{2+} dependent phospholipid binding protein with high affinity for Phosphatidyl Serine (PS). This protein is used as a sensitive probe for PS exposure upon the outer leaflet of the cell membrane and suitable for detection of apoptotic cells. The SLN loaded FOLFIRI regimen was more effective in promoting apoptotic cell death, measured by flow cytometry, than FOLFIRI

alone. Dual-parameter dot-plot of fluorescein isothiocyanate-fluorescence (x-axis) versus Propadium iodide-fluorescence (y-axis) showing fluorescence intensity (log scale). Lower left quadrants, live cells; lower right quadrants, apoptotic cells; upper left quadrants, necrotic cells; upper right quadrants, apoptotic and necrotic cells. The percentage of apoptotic cells is indicated on the plots. In HT-29 cells, SLN loaded FOLFIRI exhibited greater apoptosis, whereas FOLFIRI regimens showed reduced apoptosis.

Caspase-3

Caspase is a protease that is activated during the early stages of apoptosis and like other members of the caspase family, is synthesized as an inactive proenzyme that is processed in a cell undergoing apoptosis by self-proteolysis and /or cleavage by another protease. Active caspase-3, a marker for cells undergoing apoptosis, comprises a hetero-dimer of 17- and 112-kDa subunits, which in turn are derived from a 32-kDa pro-enzyme. Active caspase-3 proteolytically cleaves and activates other caspases and

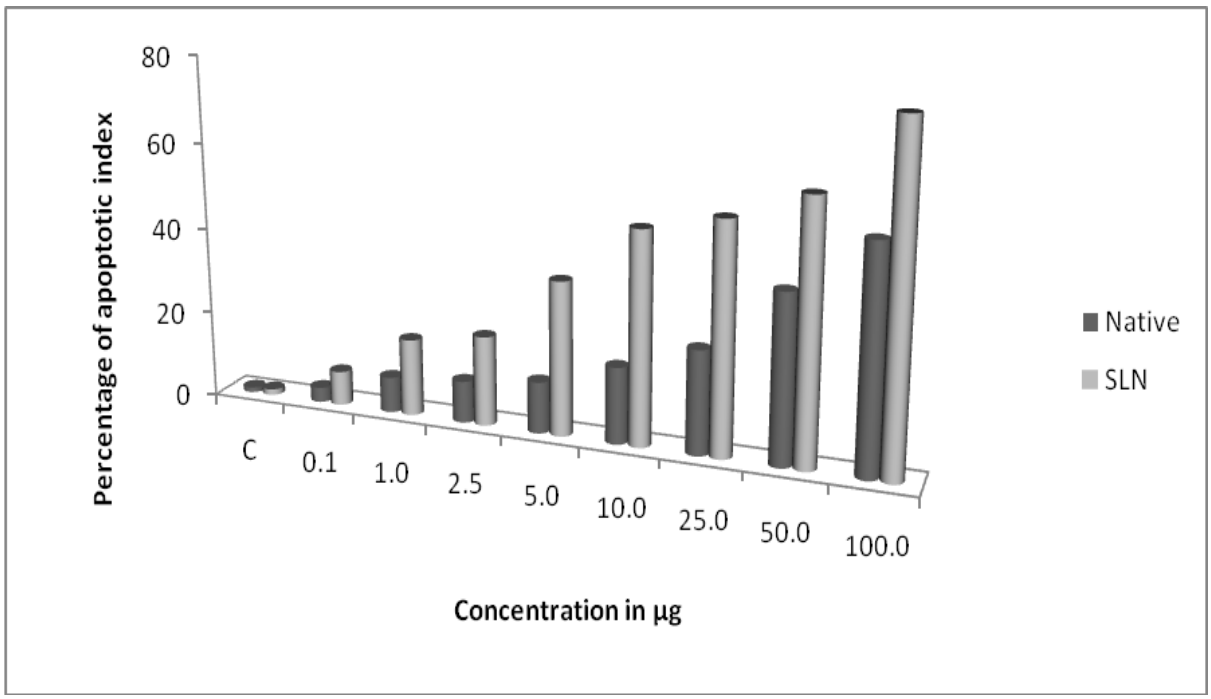
Figure 1: Flow cytometric analysis of apoptosis in the HT-29 cell line. Cells treated with the respective drug regimens and untreated controls were dual-labelled with propidium iodide (PI) and Annexin V fluorescence and analyzed by flow cytometry. (a) FSC/SSC dot plot showing the population of HT-29 cells; (b) Control cells; (c) cells treated with FOLFIRI regimen (d) cells treated with SLN loaded FOLFIRI drug regimen showing increase in the apoptotic index (the sum of the percentage of cells positive for Annexin-V-FITC alone and cells positive for both Annexin-V-FITC and propidium iodide (PI) within a population of cells)



relevant targets in the cytoplasm. We measured the caspase-3 activity in drug-treated cells. Apoptotic index as determined through Active caspase-3 assays in HT-29 cells under control was 1.1%. After treatment of cancer cell line with native drug incubating for 6 hours the percent apoptosis was 3.4 % to 51 %. The percentage of apoptosis of SLN loaded drug in HT-29 cells for different time periods was 7.9% to 76.7% (Figure 2).

Various chemotherapeutic drugs mediate their cytotoxic effects by initiating apoptosis, which is usually thought to be a non-inflammatory, non-immunogenic process. Still, it has recently been suggested that apoptosis can trail biochemically distinct subroutines, some of which may result in immunogenic cell death, in spite of the morphological consistency of apoptotic cell death. Successful drug treatment in cancer needs a satisfactory therapeutic index reflecting the treatment's specific effects on target cells and its lack of clinically significant effects on the host. In cancer, the therapeutic goal is to activate tumor-selective cell death. The systems responsible for such death are of obvious significance in determining the efficiency of specific treatments (Sellers and Fisher, 1999). The SLN loaded FOLFIRI regimen was more efficient in promoting apoptotic cell death, measured by flow cytometry, than conventional FOLFIRI. SLN loaded drug regimens showed prominent activation of caspase-3, a prevalent caspase that is ultimately responsible for the majority of apoptosis processes.

Figure 2: Exposure of Native FOLFIRI and SLN loaded FOLFIRI drug induces apoptosis in HT-29 cells. Percentage of HT-29 cells showing caspase-3 activation following treatment with drugs at concentrations ranging from 0.1 to 100 μgs in cells after 6 h incubation period



CONCLUSION

Nanotechnology has proved to be very efficient in treating cancer and is much safer than the usual chemotherapy. SLN are new category of drug carrier systems having capability to encapsulate both lipophilic and hydrophilic anticancer drugs. The outcome proved that their superiority over conventional drug formulations. The rationale design of nanoparticles for chemotherapeutic drug delivery has enabled the improved solubilization of the drug, as well as increased its stability and half-life in circulation.

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