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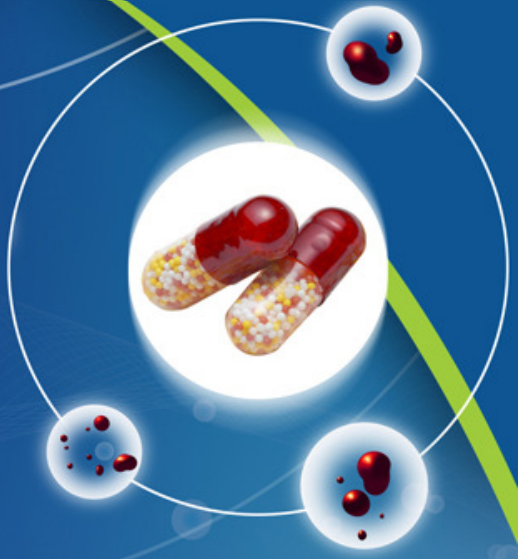

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Research Article

THE DAWNING ERA OF NANOPARTICLE ENCAPSULATED NOVEL AND PIONEERING FORMULATIONS FOR CANCER DRUG DELIVERY.

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ABSTRACT

Chemotherapy represents a core in the treatment of pancreatic cancer. Moreover, the early recognition and treatment of cancer remains a scientific bottleneck. There is an urgent need to develop new and innovative technologies that could help to delineate tumor margins, identify residual tumor cells and determine whether a tumor has been completely removed or not. Nanotechnology has witnessed significant progress in the past few decades, and its effect is widespread nowadays in every field. Nanoparticles can be modified in numerous ways to prolong circulation, enhance drug localization, increase drug efficacy, and potentially decrease chances of multidrug resistance by the use of nanotechnology. Recently, research in the field of cancer nanotechnology has made remarkable advances. This study was done to evaluate the efficacy of three nanoparticle encapsulated drugs over free drugs of the same commonly used regimens. Measures of anti-carcinogenic potential of the nano-engineered formulations were investigated using cultured carcinoma cells. Evaluation of apoptosis by Caspase-3 assay following 96 h treatment with SLN and native drugs delineated significant differences, establishing better potential efficacy of nano engineered drugs. These preliminary in vitro results suggest that SLN could be anticipated as alternative drug delivery system.

Keywords: *Cancer, chemotherapy, drug delivery, nanotechnology, solid lipid nanoparticle.*

INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer worldwide and fourth most common cause of death, with an annual incidence of approximately 1 million cases and an annual mortality of more than 500,000 [1]. Colorectal cancer is the second most widespread cause of cancer mortality among men and women [2]. Mainly colorectal cancers occur from sporadic adenomas, and a few from genetic polyposis syndromes or inflammatory bowel disease.

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The approximate ratio of colorectal cancers that is attributable to heritable causes varies from 10% to 30% [3]. The primary assumption in cancer treatment is that all malignant cells should be destroyed, removed or neutralized to achieve cure. Chemotherapy is main modalities available for the treatment of cancer. Various chemotherapeutic agents have been combined with 5-Fluorouracil (5-FU) in an attempt to enhance the single-drug response rate. A standard chemotherapy regimen widely used in colorectal cancer is composed of FOLFIRI: folinic acid (Leucovorin), fluorouracil (5-FU, Adrucil), irinotecan (Camptosar, CPT-11) [4]. In spite of recent progress in chemotherapeutic drug treatment, great numbers of demises occur each year from colorectal cancer as well as due to the adverse effects of anticancer drugs. Although chemotherapeutic agents are relatively specific,

they also kill normal cells which are dividing very rapidly such as gastrointestinal tract, bone marrow cells, and hair follicles. Side effect includes nausea, tiredness, diarrhea, stomatitis (mouth sores), loss of appetite (anorexia), loss of hair, low blood cell counts [5].

The long-term target of cancer treatment is to achieve normal life expectancy in cancer patients. On a shorter, time-period, the objectives are to increase the number of patients responding to treatment, to extend the stage of reduction, and to improve the quality of life. To attain these goals, new experimental treatment strategies should be designed based not only on research and development to improve the therapeutic potential of individual treatment modalities but also on comprehensive understanding of appropriate factors in cancer biology. Combination of drugs can be designed, using agents that produce different biochemical lesions, to attack multiple sites in biosynthetic pathways or to inhibit several processes involved in the maintenance and function of essential macromolecules. The common objective is to decrease the production and availability of a specific end product vital for tumor cell growth and replication. These limitations prompted scientists to develop novel tools to enhance drug delivery targeting cancer cells, with the expectation that improved targeting would reduce the dose required and side effects. At present, the field of nanotechnology is facilitating the delivery of chemotherapeutic drugs, by giving rise to carriers that provide for protection from degradation, prolonged circulation times, and increased tumor accumulation, all the while resulting in reduced patient morbidity.

Nanotechnology is a multidisciplinary field experiencing unpredictable progress. Nanoparticles have been developed as an important approach to deliver conventional drugs. Nanoparticles offer cell specific targeting of drugs, reduction of unwanted side effects by a controlled drug release and to deliver the drug in right site at the right time [6].. Nanoparticle defined as particles sized below 1µm and can consist out of different biodegradable materials. The drug can either be incorporated in the matrix or attached to the particle surface.

Nanoparticles prepared from solid lipids are attracting major attention as novel colloidal drug carrier for intravenous applications as they have been proposed as an alternative particulate carrier system. Several difficulties commonly encountered with anticancer compounds, such as normal tissue toxicity, poor specificity and stability and a high incidence of drug resistant tumor cells, are at least partially overcome by delivering them using SLN. SLN are sub-micron colloidal carriers ranging from 50 to 1000 nm, which are composed of physiological lipid, dispersed in water or in aqueous surfactant solution, where the drug is normally incorporated with an average diameter below 1 µm. Solid lipid nanoparticles are also referred to as "zero-dimensional" nanomaterials due to the fact that all of their dimensions are in nanoscale (under 100nm) [7]. SLN offer unique properties such as small size, large surface area, improved stability of drugs, high drug loading, controlled drug release and feasibility of incorporating both hydrophilic and hydrophobic drugs [8]. The rapid removal of colloidal particles by the macrophages of the RES is a major obstacle to targeting tissues elsewhere in the body, such as bone marrow and solid tumors. In addition, nanoparticles encapsulated with chemotherapy agents are able to avoid multi-drug resistant efflux pumps expressed on the surface of most tumor cells [9].

The main aim of the present study was to elucidate the effect of SLN loaded drugs on cancer cell line, HT-29. Evaluation of DNA cell cycle and ploidy assay through BD^{TM} Cycle TEST PLUS DNA. Evaluation of apoptotic index was done through phycoerythrin (PE) active caspase-3 assay.

MATERIALS AND METHODS

Reagents

The culture petri-dishes were procured from BD Falcon (Rockville, MD, USA). Fetal calf serum was obtained from HyClone Labs (Logan, Utah, USA). Dulbecco's Modified Eagle's Medium (DMEM) growth medium was procured from Gibco/BRL Life Technologies, Inc. (NY, USA). Antibiotic-antimycotic solution was obtained from Hi- Media Labs (Mumbai, India). The cell growth supplements sodium pyruvate, non-essential amino acids and sodium bicarbonate were obtained from MP Biomedicals, Solon, USA. SLN encapsulated drug sample was kindly gifted by Mahakal Institute of Pharmacy, Ujjain. DNA cell

cycle and ploidy were investigated using a BD™ Cycle TEST PLUS DNA Reagent Kit, BD Biosciences, USA. The activity of caspase-3 was determined by using PE Active Caspase-3 Apoptosis kit from BD™ Biosciences, San Diego, CA, USA.

Cell line & culture conditions

The human colorectal adenocarcinoma cell line, HT-29, was obtained from the National Centre for Cell Science (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, 1.5 g/L sodium bicarbonate at 37°C in the humidified atmosphere of 5% CO_2 in air 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

Cells were treated with a fixed 0.005 µM concentration of native drugs (5- Fluorouracil, Ca-leucovorin and Irinotecan) and SLN loaded (5-Fluorouracil, leucovorin and irinotecan) at different sampling intervals for time course studies ranging from 6 to 96 h.

Analysis of DNA ploidy

Analysis of nuclear DNA cell cycle was performed by staining the cultured cells with Propidium Iodide (PI) using Cycle TEST PLUS DNA Reagent kit as per the manufacturer's protocol. PI fluorescence was measured through BD™ FACS Calibur 6.0. A sum total of 30,000 events were acquired and subjected for analysis by ModFit LT 3.0 (Verity Software House Inc., Topsham, ME, and USA). Histogram displays were overlaid with graphical representations of the modelled G0/G1, S and G2/M populations following ModFit-analysis and data were expressed as percentages of cells for any given phase of the cell cycle.

Apoptotic response through PE active caspase-3 assay

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

Analysis of DNA ploidy

Treated cells showed early arrest in cell cycle progression at 12 and 48 h through G1 check point activation. Interestingly, aneuploid cells were observed at 48 h which further increased after 96 h of exposure with a distinct peak suggesting perturbation in ploidy levels due to SLN loaded FOLFIRI drugs.

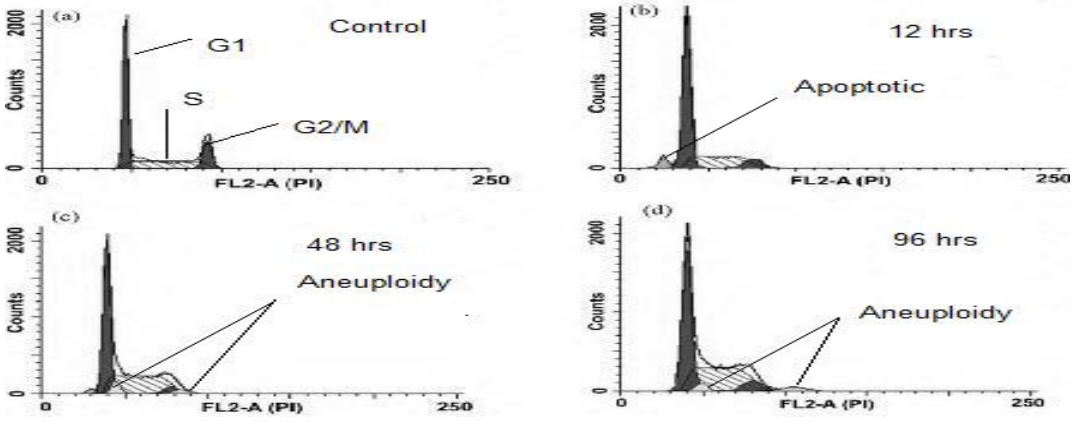

Figure 1 DNA ploidy analysis of SLN loaded FOLFIRI drugs in HT-29 cell after 96 h exposure (a) Control showing percentage of cells in G1, S and G2/M phases of the cell cycle respectively, (b) Cells after 12 h treatment showing significant arrest in G1 phase and apoptotic phase (c) a distinct sub-G1 peak showing onset of aneuploidy in cells after 48 h treatment, (d) cells after 96 h treatment showing significant increase in aneuploidy with considerable G1 arrest.

PE active caspase-3

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 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 1.4%. Post treatment of colorectal cancer cells with native drug incubating for different time periods the percent apoptosis was 5.4 % to 56 %. The percentage of apoptosis of SLN loaded drug in HT-29 cells for different time periods was 8.8% to 98.9% (Fig 2).

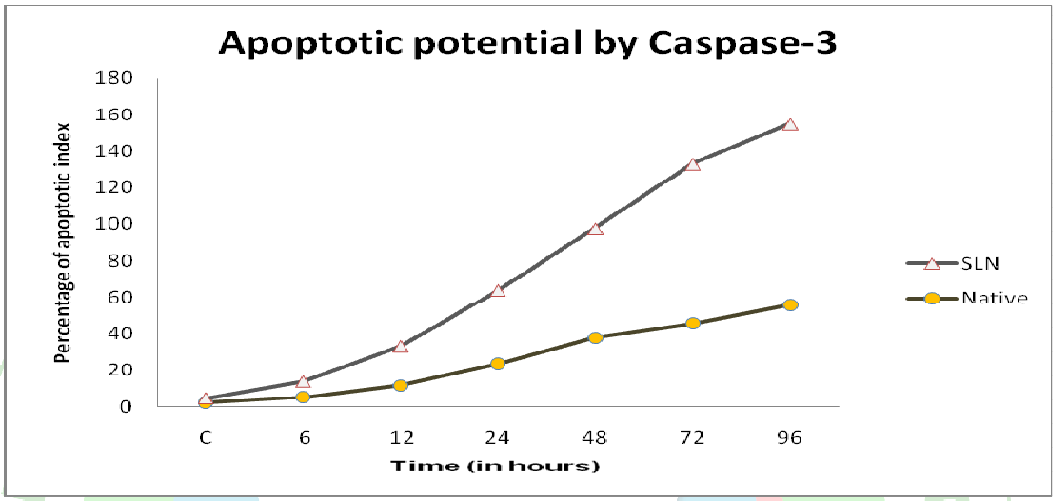

Figure 2 Apoptotic index of native and SLN loaded FOLFIRI anticancerous drug on HT-29 cells. Percentage of colorectal cancer cell showing caspase-3 activation following treatment with native and SLN loaded drugs after 6, 12, 24, 48, 72, 96 hours incubation.

DISCUSSION

Successful drug treatment in cancer needs an adequate 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 mechanisms responsible for such death are of obvious importance in determining the efficacy of specific treatments [10]. 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 is a marker for cells undergoing apoptosis. Furthermore, initiation of cell cycle arrest and apoptosis in nanoparticle encapsulated exposed cells strengthens reports that the cellular response to DSBs activates DNA damage response networks, initiating cell cycle arrest and apoptosis. An important arrest in G1 phase of the cell cycle and a main G2/M phase arrest of cells thereafter after 48 h of treatment occurred. A time dependent increase in apoptotic index in the treated cells is evidence for increased susceptibility to apoptosis on exposure.

Nanotechnology has proved to be very effective in treating cancer and is much safer than the usual chemotherapy. From the last two decades several chemotherapeutic agents have been encapsulated in SLN and their in-vitro and in-vivo efficacy have been evaluated [11]. SLN are new class of drug carrier systems having ability to incorporate both lipophilic and hydrophilic anticancer drugs. A number of anticancer drugs loaded SLN have been formulated and tested for cytotoxic studies, pharmacokinetic parameters, biodistribution studies and compared them with conventional drug formulations. Results

showed that their superiority over conventional formulations.

CONCLUSION

Cancer is a class of disorders characterized by abnormal growth of cells that proliferate in an uncontrolled way and a major disadvantage of anticancer drugs is their lack of selectivity for tumor tissue, which causes severe side effects and results in low cure rates. Thus, it is very hard to target the abnormal cells by the conventional method of the drug delivery system. Nanotechnology platforms can offer the exclusive place within this space by facilitating multimodal delivery with a single application. Although SLN's may be used for drug targeting, when reaching the projected diseased site in the body the drug carried needs to be released. So, for drug delivery biodegradable nanoparticle formulations are needed as it is the goal to carry and release the drug in order to be successful.

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