Chapter IX

Anticancer activity of the nanocomposites towards the growth of human hepatocellular liver carcinoma cell line (HepG2 cells)

9.1 Introduction

There is tremendous interest in integrating nanotechnology with medicine, creating the so-called nanomedicine with ultimate motive of diagnostic and therapeutic scope with unprecedented precision and efficiency [1]. Nanomedicines are drugs- or imaging agent-containing carriers or devices with size ranging from a few to several hundred nanometers [2]. Although the term nanomedicine emerged of late [1, 3], nanotechnology has been employed in drug delivery for decades [4]. In principle, nanomedicines are designed to enable the delivery of small molecules or macromolecular therapeutics to achieve enhanced and reorganized disease treatment by circumventing various physiological barriers. The physiological barriers may prohibit the efficient permeation of nanomedicines with undesired sizes and surface properties. Therefore, there have been substantial efforts on controlled formulation of nanomedicines. The majority of current nanotechnology platforms for chemotherapy have involved repackaging of traditional anticancer agents into various forms of nanometer-sized delivery vehicles, such as monomeric polymer–drug conjugates with sizes typically 10nm or less [2], polymeric nanoparticles [5] or self-assembled amphiphilic block-copolymer micelles [6] in a size range of 20–100 nm, or lipid [7] and polymeric vesicles [8] also known as liposomes and polymersomes, respectively with sizes between sub-100 nm to submicrometers.

There have been colossal efforts of designing nanomedicines aiming for targeted delivery of therapeutics for improved treatment of cancer, cardiovascular diseases, and immunological diseases [9-11]. One of the key challenges is the design and formulation of clinically relevant, targeted nanomedicines [11]. Many nanomedicine platforms have been developed and used in targeted drug delivery applications, including dendrimers, liposomes, polymeric nanoparticles, micelles, protein nanoparticles, ceramic nanoparticles, viral nanoparticles, metallic nanoparticles, and carbon nanotubes [10]. To facilitate the clinical application of targeted nanomedicines, their formulation should involve the use of biocompatible materials and should be completed via simple, robust processes for the
assembly of nanomedicine, fusion of drug and targeting ligand, and purification, post formulation processing, large-scale preparation, sterilization, and storage.

9.2 Anticancer activity of nanocomposites ZnO-CdS, ZnO-ZnS, SnO₂-ZnS and SnO₂-CdS that inhibits the growth of human hepatocellular liver carcinoma cell line (HepG2 cells)

9.2.1 Materials and methods

9.2.1.1 Cell culture

The HepG2 are perpetual cell lines (Human hepatocellular liver carcinoma cell line) that were obtained from the National Center for Cell Science (Pune, India) and grown in Dulbecco’s Modified Medium (DMEM) supplemented with 10 % fetal bovine serum (FBS), penicillin (100 U/ml)/streptomycin (100 µg / ml), 2 mM glutamine and 1 mM sodium pyruvate. HepG2 cells were cultured as adherent monolayers and maintained at 37 °C in a humidified atmosphere of 5 % CO₂ and 95 % air in 100 % relative humidity. HepG2 cells were harvested after brief trypsinization. All cell cultures reagents were purchased from Himedia, Mumbai, India.

9.2.1.2 Cell growth inhibition study using the MTT assay

The cell growth inhibition of nanocomposites ZnO-CdS, ZnO-ZnS, SnO₂-ZnS and SnO₂-CdS were analyzed using the 3-(4, 5-dimethylthiazol-2-yI)-2, 5- diphenyltertrazolium bromide (MTT) assay. Briefly, HepG2 cells were seeded in 96 well plates at a density of 6x10³ cells per well. After treatment with 20, 40, 80, 100, 140 and 180 µg/ml of nanocomposites ZnO-CdS, ZnO-ZnS, SnO₂-ZnS and SnO₂-CdS for 48 hours, 20 µl MTT (5 mg/ml) was added. Four hours later, 100 µl DMSO was added to each well to dissolve the resulting formazan crystals. Absorbance was read at 490 nm using an enzyme-linked immunosorbent assay reader (SpectraMax; Molecular Devices, Sunnyvale, CA).

Data were collected from triple separate experiments and the percentage of induced cell growth inhibition was determined by comparison to DMSO-treated control cells [14]. For statistical analysis, data were analyzed using analysis of variance. Fisher’s least significant difference (LSD) [13] at the 5 % level was calculated using the statistical package for social science (Version 12.0 for Windows, SPSS Inc.) to compare the means of nanocomposites ZnO-CdS, ZnO-ZnS, SnO₂-ZnS and SnO₂-CdS.
9.2.1.3 Real-Time PCR

Based on the percentage of cell growth inhibition, the mRNA expression levels of apoptotic genes; p53, bax, bcl2 in response to nanocomposites ZnO-Cds, ZnO-ZnS and SnO$_2$-ZnS a exposure in HepG2 cells (Human hepatocellular liver carcinoma cell line) were studied because apoptosis is controlled through these pathways. The primers used in this study are listed in Table 9.1. The total RNA (0.5 µg) was extracted from the HepG2 cells with TRIzol reagent. The RNA concentration was measured for each sample using a spectrophotometer at an absorbance of 260 nm. RNA samples were treated with one unit DNAse to remove any residual genomic DNA contamination. To assess mRNA expression, a reverse transcription polymerase chain reaction was done. For the RT reaction, 1 µg of total RNA was primed with oligo (dT) primer and reverse-transcribed with Expand reverse transcriptase (Roche, Basel Switzerland) in a 20 µl reaction volume.

The newly synthesized cDNA was subjected to amplification using the IQTM SYBR Green Supermix (Bio-Rad) for the real time PCR, following the manufacturer’s instruction. Amplification for each of the above genes was performed in a 20 µl reaction mixture containing 1X power SYBR Green PCR master mix, 300 nM each of forward and reverse primers and 2 µl of target cDNA. Conditions for thermal cycling were 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 sec; annealing, extension and fluorescent reading at 60 °C for 60 sec. Amplification and detection were performed with the step one real time PCR system (Applied Biosystems, California, USA). Relative gene expression was expressed as a ratio of the expression level of the gene of interest to that of GAPDH with values in untreated HepG2 cells defined as 100 %. Primer sequences were designed by primer express software (Applied Biosystems, California, USA). A negative control was included in each run and the specificity of amplification reaction was checked by melting curve analysis by the software equipped with an instrument.
<table>
<thead>
<tr>
<th>Primers</th>
<th>Primer Sequence</th>
<th>PCR Product (bp)</th>
<th>References</th>
</tr>
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<tr>
<td>P53</td>
<td>F-5’- ACT AAG CGA GCA CTG CCC AA-3’ R-5’ -ATG GCG GGA GGT AGA CTG AC-3’</td>
<td>231</td>
<td>[16]</td>
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<td>Bax</td>
<td>F-5’- CAA ACT GGT GCT CAA GGC C-3’ R-5’- GCA CTC CCG CCA CAA AGA T-3’</td>
<td>188</td>
<td>[16]</td>
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<tr>
<td>Bcl2</td>
<td>F-5’- ATG TGT GTG GAG AGC GTC AAC C-3’ R-5’- TGA GCA GAG TCT TCA GAG ACA GCC-3’</td>
<td>196</td>
<td>[16]</td>
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<td>GAPDH</td>
<td>F-5’- ACC CAC TCC TCC ACC TTT G-3’ R-5’-CTC TTG TGC TCT TGC TGG G-3’</td>
<td>178</td>
<td>[17]</td>
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Table 9.1 Primers used for real-time PCR

9.3 Result and Discussion

9.3.1 Treatment of nanocomposites ZnO-CdS, ZnO-ZnS, SnO2-ZnS and SnO2-CdS inhibits the growth of HepG2 cells

HepG2 cells were treated with different concentrations of (20, 40, 80, 100, 140 and 180 µg/ml) nanocomposites and their growth were monitored. The nanocomposites ZnO-CdS, ZnO-ZnS, SnO2-ZnS and SnO2-CdS decreased the viability of HepG2 cells in a dose-dependent manner. The maximum inhibition of cell growth (54 % with IC50) was observed at the concentration of 180 µg/ml of nanocomposites ZnO-CdS and whereas inhibition of cell growth (49 %, 44 % and 39 % with IC50) was observed at the concentration of 180 µg/ml of nanocomposites ZnO-ZnS, SnO2-ZnS and SnO2-CdS respectively.

The decreased growth rates of HepG2 cells of about 4 %, 7 %, 13 %, 20 %, 29 % and 54 % were observed at the nanocomposites ZnO-ZnS concentration of 20, 40, 80, 100, 140 and 180 µg / ml respectively (Figure. 9.1). Whereas nanocomposite SnO2-ZnS showed the growth rates of HepG2 cells of about 3.5 %, 5 %, 10 %, 13 %, 23 % and 49 % (Figure 9.2) and also nanocomposite ZnO-CdS showed growth rates about 2.5 %, 4.7 %, 9.8 %, 12.5 %, 20 % and 44 % (Figure. 9.3). However the nanocomposite SnO2-CdS showed inferior response the growth rates of HepG2 cells of about 2.1 %, 4.9 %, 11 %, 13 %, 17 % and 22 % at the concentration of 20, 40, 80, 100, 140 and 180 µg/ml respectively (Figure 9.4). All the
concentrations used in the experiment (Nanocomposites ZnO-CdS, ZnO-ZnS, SnO$_2$-ZnS and SnO$_2$-CdS) decreased the cell viability significantly (P<0.05) in a concentration-dependent manner.

9.3.2 Nanocomposites ZnO-CdS, ZnO-ZnS, SnO$_2$-ZnS and SnO$_2$-CdS induces apoptosis of HepG2 cells

Treatments with nanocomposites ZnO-CdS, ZnO-ZnS, SnO$_2$-ZnS and SnO$_2$-CdS (180 µg/ml) effectively decreased the total number of HepG2 cells and were accompanied by cell shrinkage, condensed nuclei, blebbing and shape changes (Figure 9.5). When the cells were stained, which can discriminate between apoptosis and necrosis, condensed nuclei, blebbing, indicators of typical apoptosis manifestation were observed in ZnO-CdS, ZnO-ZnS, SnO$_2$-ZnS and SnO$_2$-CdS treated cells.

Nanocomposite ZnO-ZnS treated HepG2 cells exhibited more than 54% increase in apoptosis and whereas nanocomposites SnO$_2$-ZnS, ZnO-CdS and SnO$_2$-CdS treated HepG2 cells exhibited 49%, 44% and 39% increase in apoptosis respectively and investigation of the staining pattern indicated that the predominant cause of cell death in HepG2 was due to apoptosis. Based on the one-way ANOVA analysis compared the means of nanocomposites ZnO-ZnS, SnO$_2$-ZnS, ZnO-CdS and SnO$_2$-CdS induces apoptosis of HepG2 cells significantly (P<0.05) in a concentration-dependent manner. Nanocomposite ZnO-ZnS showed higher activity compare to SnO$_2$-ZnS, ZnO-CdS and SnO$_2$-CdS treated HepG2 cells.

9.3.3 Apoptotic effect of ZnO-CdS, ZnO-ZnS, SnO$_2$-ZnS and SnO$_2$-CdS on normal cells

To test the effect of nanocomposites ZnO-ZnS, SnO$_2$-ZnS, ZnO-CdS and SnO$_2$-CdS induced similar cell death in normal HepG2 cells. After 30 min of incubation with of ZnO-ZnS, SnO$_2$-ZnS, ZnO-CdS and SnO$_2$-CdS (180 µg/ml) resulted only 15.3% cell death. Concentrations used in the experiment (ZnO-ZnS, SnO$_2$-ZnS, ZnO-CdS and SnO$_2$-CdS) decreased the cell viability were not significantly (P<0.05) in a concentration 180 µg/ml.
Figure 9.1: Cytotoxic effect of ZnO-ZnS Nanocomposite against HepG2 (Human hepatocellular liver carcinoma cell line)

Figure 9.2: Cytotoxic effect of SnO$_2$-ZnS Nanocomposite against HepG2 (Human hepatocellular liver carcinoma cell line)
Figure 9.3: Cytotoxic effect of ZnO-CdS Nanocomposite against HepG2 (Human hepatocellular liver carcinoma cell line)

Figure 9.4: Cytotoxic effect of SnO$_2$-CdS Nanocomposite against HepG2 (Human hepatocellular liver carcinoma cell line)
Figure 9.5: Cytotoxicity and apoptotic activity of Nanocomposites of ZnO-CdS, ZnO-ZnS and SnO$_2$-ZnS against HepG2 (Human hepatocellular liver carcinoma cell line). (A) Controls cells; (B) ZnO-CdS treated HepG2 cells; (C) ZnO-ZnS treated HepG2 cells; (D) SnO$_2$-ZnS treated HepG2 cells showing cytotoxicity and apoptotic activity.

9.3.4 Relative apoptotic gene expression of Nanocomposites ZnO-CdS, ZnO-ZnS and SnO$_2$-ZnS treated HepG2 cells

9.3.4.1 Isolation of mRNA and cDNA synthesis

Total RNA was extracted from HepG2 cells and the quality assessment of RNA extracted from HepG2 cells was done by agarose gel electrophoresis (Figure 9.6). Lane 2 and 3 shows the presence of the tow intact ribosomal RNA bands (28S and 18S) with the intensity of the 28S ribosomal band approximately twice that of the 18S band. This is the indirect indication that the mRNA is intact without degradation in this preparation from HepG2 cells. To purify the mRNA form this total RNA, 250 µg of total RNA was used (Figure 9.7). Lane
2 shows mRNA purified using mRNA mini purification kit. The total mRNAs appeared as a continuous smear from 500 bp to >5 kb. Appearance of intact residual 28S and 18S ribosomal RNA indicated the intact and enrichment of mRNA. Distribution of mRNA centered in the 1-3 kb region indicated that the majority of the mRNAs are in this size range. The continuous smear of mRNA and the persistence of intact rRNAs indicated that the mRNA is not degraded. The purity of RNA preparation was assessed by spectrophotometric analysis prior to reverse transcription. Purity of the RNA was 2.0 at the ratio of the 260/280 nm. This results indicated the extracted RNA and mRNA are intact and of high quality for cDNA synthesis to assess mRNA expression.

For cDNA synthesis a reverse transcription polymerase chain reaction was done. For the RT reaction, 1 µg of total RNA was primed with oligo (dT) primer and reverse-transcribed with Expand reverse transcriptase (Roche, Basel Switzerland) in a 20 µl reaction volume. The newly synthesized cDNA (Figure 9.8) was subjected to amplification using the IQTM SYBR Green Supermix (Bio-Rad) for the real time PCR.

![Figure 9.6: Agarose gel electrophoresis of HepG2 cells RNA. Lane 1- Marker, Lane 2-4 total RNA from HepG2 cells treated by nanocomposites ZnO-CdS, ZnO-ZnS and SnO$_2$-ZnS](image)

Figure 9.6: Agarose gel electrophoresis of HepG2 cells RNA. Lane 1- Marker, Lane 2-4 total RNA from HepG2 cells treated by nanocomposites ZnO-CdS, ZnO-ZnS and SnO$_2$-ZnS
Figure 9.7: Agarose gel electrophoresis of HepG2 cells RNA. Lane 1- Marker, Lane 2 mRNA purified from total RNA of HepG2 cells treated by nanocomposites.

Figure 9.8: Agarose gel electrophoresis of HepG2 cells cDNA synthesis. Lane 1- 3 cDNA of HepG2 cells treated by Nanocomposites ZnO-CdS, ZnO-ZnS and SnO$_2$-ZnS treated.

9.3.5 Relative apoptotic gene expression of Nanocomposites ZnO-CdS, ZnO-ZnS and SnO$_2$-ZnS treated HepG2 cells

The molecular mechanism of nanocomposites ZnO-CdS, ZnO-ZnS and SnO$_2$-ZnS induced apoptosis in HepG2 cells, the expression levels of apoptosis-related genes were examined. The relative quantification of $p53$, $Bax$ and $Bcl2$ mRNA expression levels was performed by SYBR Green-based quantitative real-time PCR (Applied Biosystems, USA).
HepG2 cells were exposed to ZnO-CdS, ZnO-ZnS and SnO₂-ZnS nanocomposites at a concentration of 180 µg/ml for 18 h, 36 h and 48 h. It showed that the mRNA levels of these apoptotic markers were significantly altered in HepG2 cells due to ZnO-CdS, ZnO-ZnS and SnO₂-ZnS nanocomposites exposure. The mRNA level of 18 h and 36 h of ZnO-ZnS of nanocomposite treated cells shows the tumour suppression gene p53 was up-regulated 1.695 and 2.893 fold at 18 h and 36 h and 3.347 fold at 54 h higher in treated cells as compared to the control which is shown in the Figure 9.9. The mRNA level of 18 h and 36 h ZnO-CdS and SnO₂-ZnS nanocomposites treated cells showed the tumor suppression gene p53 was up-regulated. The Figure 9.10 and 9.11 shows that p53 level of up-regulation is less when compared to ZnO-ZnS nanocomposite (Table 9.2).

Figure 9.9: Real time PCR analysis of ZnO-ZnS of nanocomposite treated HepG2 cells shows, up regulation of p53 and Bax genes and down regulation of Bcl-2 genes.
Figure 9.10: Real time PCR analysis of ZnO-CdS of nanocomposite treated HepG2 cells shows, up regulation of \( p53 \) and \( Bax \) genes and down regulation of \( Bcl-2 \) genes.

Figure 9.11: Real time PCR analysis of SnO\(_2\)-ZnS of nanocomposite treated HepG2 cells shows, up regulation of \( p53 \) and \( Bax \) genes and down regulation of \( Bcl-2 \) genes.
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<th>ZnO-CdS 54h</th>
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<td>-2.698</td>
<td>-1.061</td>
<td>-1.345</td>
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Table 9.2: Real time PCR analysis of ZnO-CdS, ZnO-ZnS and SnO₂-ZnS of Nanocomposites treated HepG2 cells.

9.4 Conclusion

Large scale screening of nanocomposites on cell culture is an important initial step to determine their potential efficacy in clinical application. Several reports have shown that nanocomposites replicate at tumor sites under hypoxic conditions and stimulate the host immune response and gene expression, leading to the inhibition of tumour growth. Nanocomposites were successfully synthesized using hydrothermal method. The present study explores the potential anticancer activity of synthesized ZnO-CdS, ZnO-ZnS, SnO₂-ZnS and SnO₂-CdS nanocomposites on human hepatocellular liver carcinoma cell line (HepG2 cells). The cytotoxic and genotoxic mechanism of nanocomposites is related to their membrane penetration and apoptosis activity. An induction of apoptosis in cancer cells is considered as a new focus in the discovery of anti-cancer drugs. Cell cycle arrest analysis for ZnO-CdS, ZnO-ZnS, SnO₂-ZnS and SnO₂-CdS nanocomposites revealed the influence of nanocomposites nanoparticles on the apoptosis of HepG2 cells. The cytotoxic effect of zinc oxide-zinc sulphide nanocomposite was relatively higher when compared to other nanocomposites.

References