

Chapter 7 In vitro cell line studies of developed TNPs, HA-TNPs and CS-TNPs

7.1 Materials

Temozolomide (TMZ) was obtained as a gift sample from Cipla Ltd., Mumbai (India). Cell culture plates (96, 12 and 6 well plates), culture flasks, Dulbecco's Modified Eagle Medium (DMEM), Hank's Balanced Salt solution (HBSS), fetal bovine serum (FBS), Trypsin-EDTA, penicillin-streptomycin solution (100 U/ml), fluorescein isothiocyanate (FITC), 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenyl-formazan (MTT) were purchased from Himedia (India). Transwell inserts were purchased from Corning, NY, (USA). Micropipettes were purchased from Thermo fishier Scientifics. Micro tips were purchased from Tarson (India). All other chemicals and solvents used were of analytical grade. The U-373 MG, U-87 MG and MDCK II cell lines were purchased from National Center for Cell Science (NCCS) Pune (India). Refer section 6.1 for sources of materials used for the preparation and surface modification of HA-TNPs and CS-TNPs.

7.2 Equipments

- Digital analytical balance (ATX224 Shimadzu, Japan)
- Cooling centrifuge (Remi equipment Pvt Ltd, India)
- Magnetic stirrer (Remi sci. Equipment, India)
- Deep freezer (EIE Inst. Ltd, Ahmedabad)
- Sonicator: Modern Industrial Corporation (Mumbai, India)
- Fluorescence Microscope: FSX100 (Olympus, USA)
- Flow Cytometer: BD FACS Aria III (BD Biosciences, CA)
- BD FACS Diva software version 6.1.3
- Fluorescence Microplate Reader: Fluoroskan Ascent CF (Labsystems, USA)
- Confocal Microscope: Zeiss LSM 510 confocal microscope (Germany)
- ELISA Plate reader: Bio-Rad, Hercules, CA, USA

7.3 Methods

Chapter 7 In vitro cell line studies of developed TNPs, HA-TNPs and CS-TNPs

7.3.1 Cell culture protocols

The U-87 MG cells were obtained from the cell repository facility of NCCS, Pune, India. The cell lines were maintained at 16h prior to the experiments as monolayer cultures in DMEM media supplemented with 10% heat inactivated fetal bovine serum (FBS) and 1% antibiotic (streptomycin + penicillin). Cultures were maintained at 37°C in a humidified 5% CO₂ atmosphere. The protocols for expanding the cell culture and counting the cells are based on the protocols and methodology described in Culture of Animal Cells: A Manual of Basic Technique and Specialized Applications, 6th Edition, Ed: Freshney R. I., Wiley- Blackwell Publication, 2010 (1)

7.3.1.1 Expanding the cell culture

- 1) DMEM and HBSS were warmed up in the water bath at 37°C.
- 2) The cell growth of the cells was examined under the microscope. For the experiments the confluence of cells should be ~80%.
- 3) The medium of ~80% confluence flask was removed with the help of micropipette and HBSS (5 ml) was added to non-cell side of the flask and allowed to stand on cell side.
- 4) HBSS was removed from the flask with the help of micropipette (away from cells) and trypsin (1 ml) was added to the flask (on non-cell side) and swirled occasionally for detachment of cells. Then DMEM (5 ml) was added to the flask.
- 5) The fluorescence microscope (FSX-100, Olympus, USA) was used to examine the cell detachment.
- 6) The Trypsin/medium/cell suspension was collected in a test tube (15 ml) and centrifuged at 600 rpm for 6 min for settling the cells (as pellet) at the bottom of the test tube.
- 7) The supernatant was removed with the help of micropipette.
- 8) Then DMEM (10 ml) was added to the test tube and mixed.
- 9) The cell suspension was splitted equally between 10 T-25 flasks (1 mL in each flask).
- 10) Then DMEM (9 ml) was added to each flask and flasks were incubated at 37°C in 5% CO₂.

Chapter 7 In vitro cell line studies of developed TNPs, HA-TNPs and CS-TNPs

7.3.1.2 Cell counting in multi-well plates

- 1) The medium was removed from cell culture plate and cells were washed with 1 mL (24 wells) of HBSS.
- 2) Then HBSS was removed and trypsin (100 μ l) was added to each well and incubated for 5 minutes. Cells were examined under the microscope.
- 3) In the next step, each well was washed with culture medium (100 μ l) and combined all solutions for measuring the volume of cells.
- 4) The cells were then mixed by gently flushing the cell/medium mixture up and down with a pipette.
- 6) Two independent 1:10 dilutions were made (if necessary) of the cell suspension.
- 7) Haemocytometer was used for cell counting (two counts from each dilution).

Note: The cells which were within two inner lines of the triple line boundary of the four 1 mm³ squares (each of these squares is made of 16 small squares) should be counted only.

$$\text{The number of cells per ml} = \left(\frac{\text{Number of cells}}{4} \right) \times 10000 \times \text{Dilution factor} \quad (\text{Equation 7.1})$$

. For example, if average count is 100 cells in all four squares and cells were undiluted:

$$\text{Number of cells/ml} = 100/4 \times 10^4 \times 1 = 25,000 \text{ cells/ml}$$

7.3.1.3 Seeding 96-well plates with cells

- 1) The medium was removed from the T-flask and flask was rinsed with sterile HBSS (6 ml).
- 2) The cells were detached from the flask using trypsin (1 ml).
- 3) After the cells detachment, DMEM media containing 10% FBS (9 ml) was added.
- 4) Then the cell suspension was transferred to centrifuge tube (50 ml).
- 5) The cell suspension was centrifuged at 1000 rpm for 7 minutes and supernatant was removed.
- 6) The cell pellet was re-suspended in the small amount of DMEM.
- 7) Sufficient medium was added till enough volume of cell suspension was achieved to seed the plate.

Chapter 7 In vitro cell line studies of developed TNPs, HA-TNPs and CS-TNPs

8) 1 ml of cell suspension was added into each well of 96-well plate and incubated at 37 °C and 5% CO₂ for 24-48 h (depending on the cell concentration).

7.3.2 *In vitro* BBB Passage Study

The permeation of TMZ, TNPs, HA-TNPs and CS-TNPs across the BBB was assessed by *in vitro* BBB model (co-culture model and monolayer model) (2). In the co-culture model, U-373 MG cells (density = 7.5×10⁴ cells/well) were seeded and grown onto the apical side of inserts. Then inserts were transferred to 12-well culture plates containing DMEM medium (1ml) and were incubated for 24 h at 37 °C. After incubation, MDCKII (density = 150×10⁴ cells/well) were seeded and incubated at 37 °C for 24 h onto the inner side of the insert. After incubation, 1 ml of TMZ, TNPs, HA-TNPs and CS-TNPs at TMZ concentration of 2 mg/ml were added to the luminal compartment of inserts. Then, 200 µl of medium was withdrawn from the basal compartment at predetermined time interval (0, 2, 24 and 48 h), replacing with fresh medium. The permeation of drug through the *in vitro* BBB model was determined by HPLC (Agilent Technologic 1260 Infinity II) analysis (2) and transport ratio of TMZ, TNPs, HA-TNPs and CS-TNPs was determined using the following equation:

$$\text{Transport ratio (\%)} = \left(\frac{W_n}{W}\right) * 100 \dots\dots\dots\text{Equation 7.1}$$

Where, W_n = amount of TMZ in the basal chamber at time “n” (n = 2, 24 and 48 h); W = amount of TMZ added in the apical chamber.

In the monolayer model, only single cell line at a time was grown in similar manner to the co-culture model and above mentioned procedure was followed.

7.3.3 *In-vitro* cell viability assay

The *in-vitro* cell cytotoxicity of the prepared nanoparticles (TNPs, HA-TNPs and CS-TNPs along with pure TMZ) against U-87 MG cells was estimated by MTT assay. Briefly, the cells were seeded in a 96-well cell culture plate at a density of 2500 cells/well and incubated for 24 h at 37 °C and 5 % CO₂ in media. After incubation, the old medium was replaced with fresh medium containing different concentrations (5-100 µg/ml) of pure TMZ, TNPs, HA-TNPs and

Chapter 7 In vitro cell line studies of developed TNPs, HA-TNPs and CS-TNPs

CS-TNPs and incubated again for 24 and 72 h, respectively. Then, MTT solution at concentration of 5 mg/ml (20 μ l) was added to the cells and were further incubated overnight in the dark at 37°C. After incubation, MTT solution was removed and 150 μ l DMSO was added to dissolve the formazan crystals. Thereafter, the absorbance was immediately measured at 570 nm by a micro-plate reader (Bio-Rad, Hercules, CA, USA) (3).

7.3.4 Cell cycle analysis

U87 MG cells (density of 1×10^5 cells/well) were seeded on plates and incubated at 37 °C for 24 h. After 24 h, old medium was discarded, replaced with fresh medium containing TMZ, TNPs, HA-TNPs and CS-TNPs at concentration equivalent to 100 μ g/ml of pure drug and incubated for another 24 h. Then cells were detached using trypsin-EDTA, washed with PBS and fixed using 70% ethanol. Staining of the fixed cells were done with 0.5 ml of PBS containing 0.5 μ g/ml propidium iodide, 10 μ g/ml RNase A and 0.1% triton X-100. The cells were incubated for 30 min at room temperature in dark and cell cycle analysis was performed using BD FACS Aria III (BD Biosciences, CA). Data was analyzed using BD FACS Diva software version 6.1.3 (4).

7.3.5 Cellular uptake study

The cellular uptake of TNPs, HA-TNPs and CS-TNPs (qualitative uptake) in U-87 MG cell line were performed using confocal microscopy. The cells (density of 5×10^3 cell/well) were grown on cover slips (coated with poly l-lysine) at 37 °C for 24 h. After 24 h, old medium was replaced and cells were treated with medium containing FITC tagged TNPs, HA-TNPS and CS-TNPs for 2 h. In the next step, cells were washed and fixed by PBS and 1% PFA respectively. The cover slips were washed thrice using PBS and mounted on glass slides using 2.5 % DABCO and sealed. Cellular uptake of FITC tagged TNPs, HA-TNPs and CS-TNPs was then analyzed using Zeiss LSM 510 confocal microscope (Oberkochen, Germany) at 63X (2).

7.3.6 Cellular uptake mechanism

The uptake poisoning method was utilized to investigate the cellular uptake and endocytosis mechanism of TNPs, HA-TNPs and CS-TNPs in U-87 MG cell monolayers. U-87 MG cells (1×10^5 cells/well) were seeded on 6-well plate and allowed to grow for 24 h. After 24h, old

Chapter 7 In vitro cell line studies of developed TNPs, HA-TNPs and CS-TNPs

media was replaced with fresh culture medium containing amiloride (25 µg/ml), chlorpromazine (10 µg/ml) and nystatin (50 µg/ml) and cell monolayers were incubated for 1 h at 37 °C. After 1 h, TNPs, HA-TNPs and CS-TNPs (25 µg/ml each) were added and co-incubated for 12 h and 24 h. After incubation, cells were lysed using 1% Triton X-100 and amount of TMZ inside the cells was analyzed by HPLC (5).

CD44 blocking assay was used to study uptake of HA-TNPs and CS-TNPs by CD44 receptor. For that, the cells were pretreated with 10 mg/ml free HA polymer (175-350 kDa and hydrated overnight in serum and antibiotic free medium) for 1 h before addition of HA-TNPs and CS-TNPs, again incubated for an additional 12 h and 24h. After incubation, cells were lysed using 1% Triton X-100 and amount of TMZ inside the cells was analyzed by HPLC (5).

7.3.7 Reactive oxygen species (ROS) generation study

Dichlorofluorescein (DCF) assay was utilized to estimate ROS generation in U-87 MG cells. The cells were seeded in 96 well plate and incubated for 24 h. After 24h, medium was replaced with fresh medium containing TMZ, TNPs, HA-TNPs and CS-TNPs (5-100 µg/ml). In this analysis, 10 µg ml⁻¹ of H₂O₂ was used as a positive control while untreated cells were used as negative control. Cells were harvested, washed once with PBS and incubated with 10 µM H₂DCFDA (2,7-dichlorodihydrofluorescein diacetate) (in PBS 1×) for 30 min at 37°C prior to analysis. The DCF fluorescence was then recorded at 535 nm using a plate reader (Fluoroskan Ascent CF (Labsystems, USA)). The generated ROS was expressed as a ratio of the fluorescence of DCF of treated cells to that of untreated cells (5,6).

7.3.8 Scratch assay

U-87 MG cells (2×10^5 cells/ well) were plated in 6-well plates and incubated at 37°C and 5% CO₂ environment to form a confluent monolayer. After monolayer formation, scratch was created with the help of p200 pipette tip. The scratch containing cells were washed with growth medium to remove the debris and smoothening of scratch. Then old media was replaced with 1 ml of fresh medium specific for the in vitro scratch assay. To obtain the same field during the image acquisition, markings were created to be used as reference points close to the scratch.

Chapter 7 In vitro cell line studies of developed TNPs, HA-TNPs and CS-TNPs

Then cells were treated with TMZ, TNPs, HA-TNPs and CS-TNPs at a concentration equivalent to 100 µg/ml of TMZ. In control group wells, no sample was added. Then plates were incubated at 37 °C and examined daily until the scratch in the control group wells was filled completely with cells (7)

7.4 Results and discussion

7.4.1 *In vitro* BBB passage/ flux study

In vitro BBB passage of pure TMZ, TNPs, HA-TNPs and CS-TNPs were estimated and results are summarized in Figure 7.1 and 7.2. The results indicated that the transport efficiency of TNPs and HA-TNPs increased in a time dependent manner (Figure 7.1). The transport ratio of HA-TNPs was more than pure TMZ and TNPs through the BBB model at all the tested time points: TMZ < TNPs < HA-TNPs which may be due to HA surface modification and indicated higher BBB passage ability of HA-TNPs as compared to pure TMZ and TNPs. The passage of the developed nanoparticles through BBB was assessed by constructing mono layer co-culture model using U-373MG cells (as glial cells) and MDCK cells (as endothelial cells). In case of monoculture, the permeation of HA-TNPs was more as compared to pure TMZ and TNPs. But, permeation of HA-TNPs was lesser in co-culture model as compared to monoculture model. This variation may be attributed to the fact that in case of monolayer model, the tightness among the junctions of the cells is not achieved. Due to improper junctions, the model allows passage of free and the trapped drug at the same rate. These results may be interesting for predicting the uptake of nanoparticles by tumor cells which have enhanced permeability and leakiness and will show greater uptake due to EPR effect. Although, the co-culture model is much more robust and mimics the *in vivo* BBB to a greater extent in normal conditions (2), it may not be suitable for assessing uptake in disease conditions like brain tumors.

In case of CS-TNPs also, the transport ratio of CS-TNPs was higher than pure TMZ and TNPs through the co-culture model at all the tested time points which indicated higher BBB passage ability of CS-TNPs as compare to pure TMZ (figure 7.2). When TNPs and CS-TNPs were compared, transport ratio was found to be more in CS-TNPs which may be due to conjugation of CS with TNPs which facilitated the transport of TNPs across BBB as apart from

Chapter 7 In vitro cell line studies of developed TNPs, HA-TNPs and CS-TNPs

CD44 receptor targeting, CS also facilitates BBB crossing (7). In case of monoculture model, similar results were seen as in co-culture model. But when transport of CS-TNPs across co-culture model was compared with monolayer model, it was found to be lesser, which may be due to formation of tight junction in co-culture model (5).

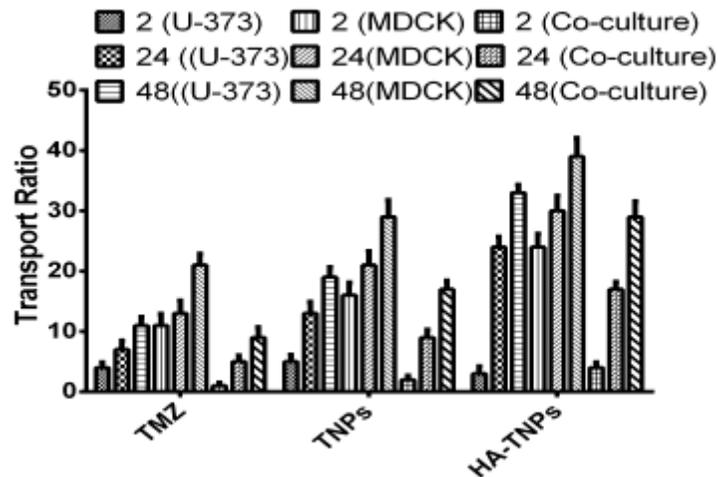


Figure 7.1: *In vitro* BBB passage studies for pure TMZ, TNPs and HA-TNPs after 2h, 24h and 48h

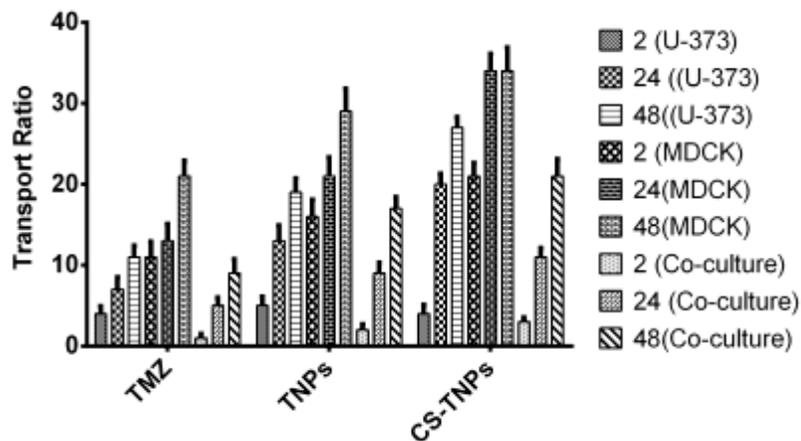


Figure 7.2: *In vitro* BBB passage studies for pure TMZ, TNPs and CS-TNPs after 2h, 24h and 48h. Data is represented as mean \pm SD (n = 3)

Chapter 7 In vitro cell line studies of developed TNPs, HA-TNPs and CS-TNPs

7.4.2 In vitro cell viability assay

MTT assay was performed to assess cell viability of TMZ, TNPs, HA-TNPs and CS-TNPs and results indicated concentration dependent suppression of cell viability. As compared to TMZ, TNPs demonstrated more than 50 % suppression of cell viability (figure 7.3A). TNPs demonstrated 49.0 ± 2.1 % cell viability at 100 $\mu\text{g}/\text{mL}$ concentration after 24 h while pure TMZ alone showed 64.0 ± 2.0 % cell viability. As compared to TMZ, TNPs showed 1.31 folds higher suppression of cell viability. The reason of higher suppression of cell viability in case of TNPs may be due to slow release and longer retention of TMZ from TNPs as compared to pure TMZ and hence cytotoxicity of TNPs was more as compared to pure TMZ (8)

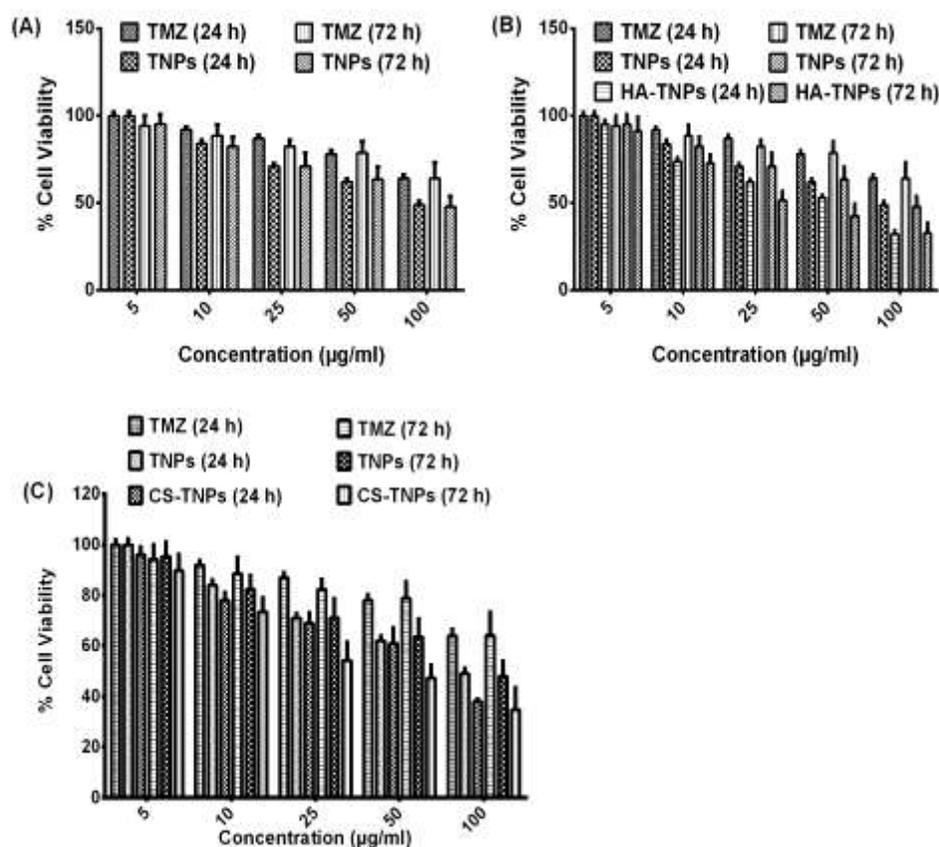


Figure 7.3: In vitro cell viability study in U-87MG cells after 24h and 72 h (A) Comparison of TMZ and TNPs, (B) Comparison of TMZ, TNPs and HA-TNPs and (C) Comparison of TMZ, TNPs and CS-TNPs. Data is represented as mean \pm SD (n = 3)

Chapter 7 In vitro cell line studies of developed TNPs, HA-TNPs and CS-TNPs

In case of HA-TNPs and CS-TNPs, as compared to TMZ and TNPs, HA-TNPs and CS-TNPs both demonstrated more than 50% suppression of cell viability. HA-TNPs and CS-TNPs demonstrated $32.8\% \pm 2.0\%$ and $38.0\% \pm 1.0\%$ cell viability at $100\ \mu\text{g/ml}$ concentration respectively after 24 h which was 1.95 folds and 1.68 folds higher respectively than TMZ (figure 7.3B and 7.3C). As compared to TNPs, HA-TNPs and CS-TNPs both showed 1.49 folds and 1.29 folds enhanced cell suppression ability respectively which may be correlated with its (HA-TNPs and CS-TNPs) higher cellular uptake and CD44 receptor targeting ability that led to higher suppression of cell growth. Apart from this, from HA-TNPs and CS-TNPs also, release of TMZ was slow and it retained longer as compared to pure TMZ and hence cytotoxicity of HA-TNPs and CS-TNPs was more as compared to pure TMZ (8). In all three developed nanoparticles (TNPs, HA-TNPs and CS-TNPs), no significant change in cell viability was seen after the incubation of cells for 72 h. This may be due to short half life of TMZ (approximately 2h) in physiological condition (pH 7.2) (3).

7.4.3 Cell cycle analysis

Cell proliferation is associated with the cell cycle and different phases (G1, G2, M and S) can be distinguished based on the DNA content measurement by propidium iodide (PI) staining. As reported earlier TMZ act on the G2/M phase of cell cycle and cause cell cycle arrest in this phase which lead to inhibit cell proliferation (4). So cell cycle analysis was done to evaluate the inhibitory potential of developed NPs on G2/M phase and cell proliferation and results are demonstrated in figure 7.4 and 7.5. In U-87 MG cells, TMZ ($100\ \mu\text{g/ml}$) induced cell cycle arrest in G2/M phase at 42.8 % which was approximately 1.17 folds higher as compared to control cells whereas TNPs induced 46.2 % accumulation of cells in G2/M phase which was 1.25 folds higher than untreated control cells. As compared to pure drug, higher accumulation of cells in G2/M phase was observed in case of TNPs. In case of HA-TNPs no significant change in G2/M phase arrest was observed (figure 7.4) as compared to untreated control cells which may be correlated with the fact that drug was not significantly release from HA-TNPs in given treatment time duration due to presence of HA which sustained the release of TMZ.

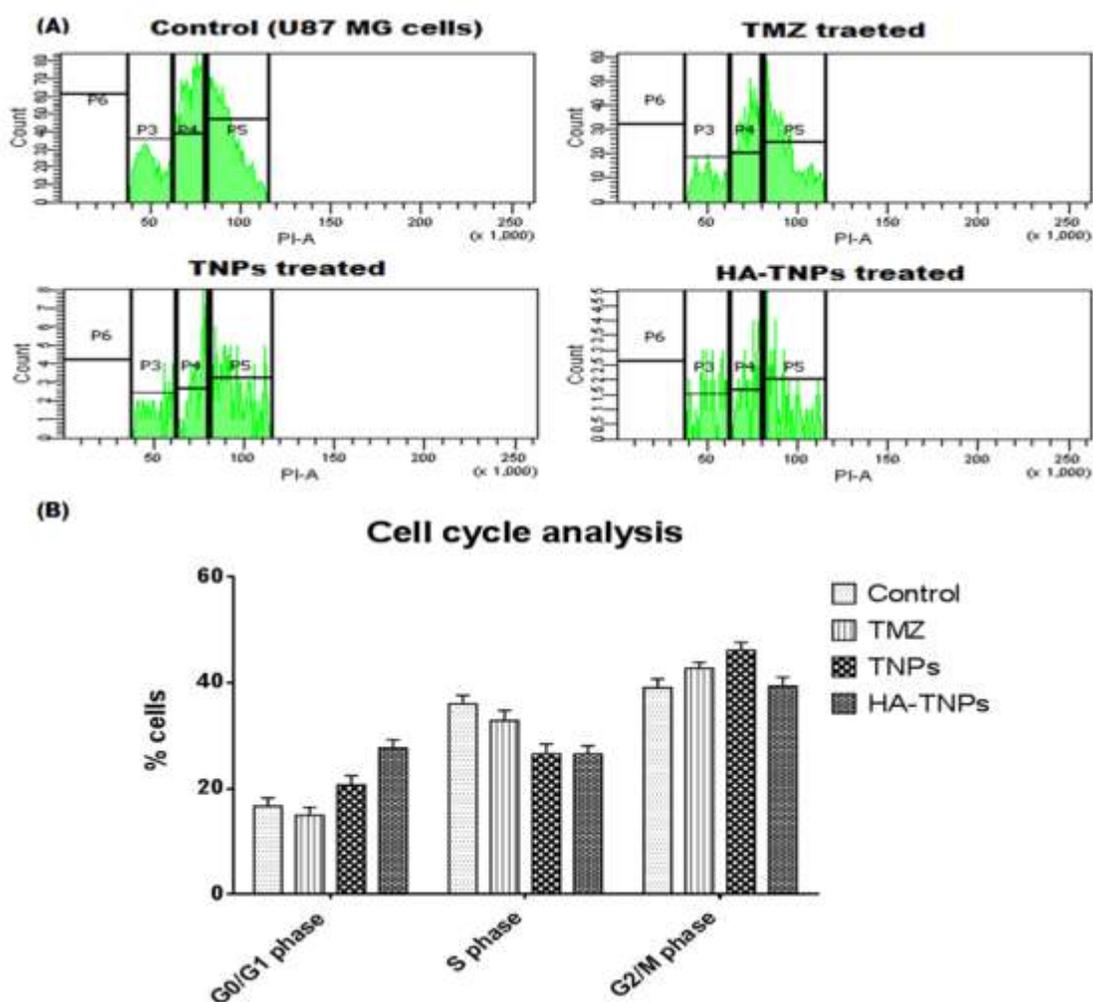


Figure 7.4: Cell cycle analysis of Control, Pure TMZ, TNPs and HA-TNPs on U-87 MG cells (A) flow cytometry analysis using propidium iodide staining for cell cycle analysis. (B) Percentage cell distribution at different phases of cell cycle for control cells, Pure TMZ, TNPs and HA-TNPs. Data is represented as mean \pm SD (n = 3)

In case of CS-TNPs, 51.81 % accumulation of cells in G2/M phase was observed (figure 7.5) which was 1.33 folds higher than untreated control cells. As compared to pure drug and TNPs, higher accumulation of cells in G2/M phase was observed with CS-TNPs which suggested that CS-TNPs caused significant inhibition of cell growth. The reason of higher cell cycle arrest with CS-TNPs may be correlated with CD44 receptor mediated higher uptake of CS-TNPs than TNPs. This higher uptake of CS-TNPs led to increased concentration of loaded TMZ inside the

Chapter 7 In vitro cell line studies of developed TNPs, HA-TNPs and CS-TNPs

cells which caused enhanced cell cycle arrest in G2/M phase and indicated higher suppression of cell growth. The observed results were also similar with previous findings in which authors reported G2/M phase cell cycle arrest by TMZ loaded nanoparticles (4).

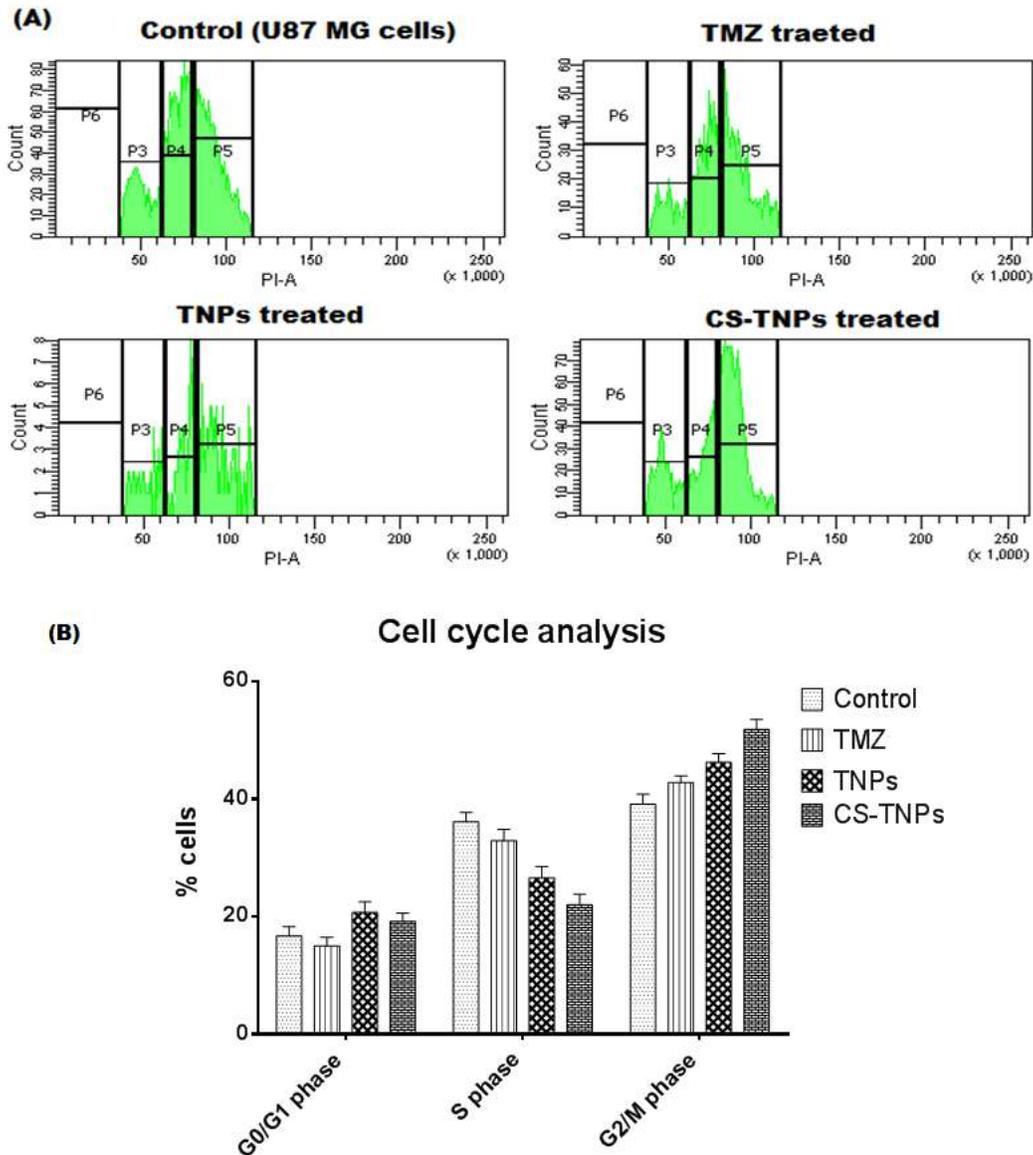


Figure 7.5: Cell cycle analysis of Control, Pure TMZ, TNPs and CS-TNPs on U-87 MG cells (A) flow cytometry analysis using propidium iodide staining for cell cycle analysis. (B) Percentage cell distribution at different phases of cell cycle. Data is represented as mean \pm SD (n = 3)

7.4.4 Cellular uptake

Qualitative cellular uptake of FITC tagged TNPs, HA-TNPs and CS-TNPs on U-87 MG cells were assessed by confocal microscopy and result is shown in figure 7.6 and 7.7. As seen in image (figure 7.6), TNPs were taken up by cells which may be due to albumin. As per the reported literature, albumin binding receptors (SPARC and gp-60) are over expressed in U87 MG cells for transportation of albumin (8) so TNPs might be bind to these receptors which enhanced the uptake of TNPs. Apart from this, albumin also triggers the endocytosis into tumors cells which was further confirmed by demonstration of cellular uptake mechanism.

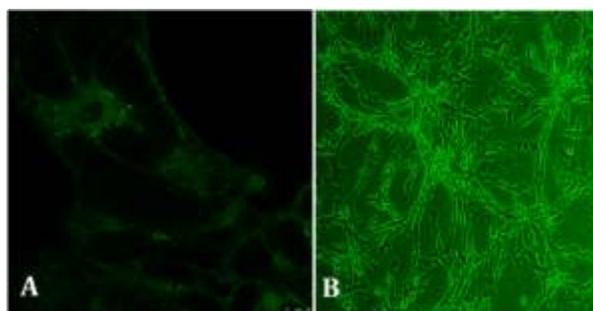


Figure 7.6: Cellular uptake of TNPs in U87 MG cells (A) and (B) untreated U-87 MG cells

In case of HA-TNPs and CS-TNPs, As seen in image (figure 7.7), uptake of HA-TNPs and CS-TNPs were more as compared to TNPs which may be due to presence of HA or CS over the surface of TNPs. Surface modification of TNPs with HA or CS may have facilitated CD44 receptor mediated uptake of CS-TNPs and led to higher uptake than TNPs (10). This was further confirmed by demonstration of cellular uptake mechanism.

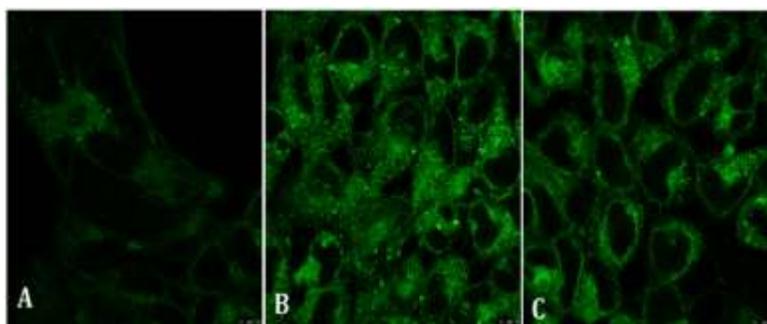


Figure 7.7: Cellular uptake of (A) TNPs, (B) HA-TNPs and (C) CS-TNPs in U-87 MG cells

7.4.5 Cellular uptake mechanism

Various receptor poisons were used to study the uptake mechanism in which chlorpromazine, nystatin, amiloride and HA were used to inhibit clathrin, caveolae, macropinocytosis and CD44 receptor mediated uptake respectively (5). Cellular uptake mechanism of TNPs, HA-TNPs and CS-TNPs across U-87 MG cell monolayer was evaluated (figure 7.8). The results demonstrated that cellular uptake of TNPs in presence of chlorpromazine was significantly reduced to $28.8\% \pm 1.4\%$ and $25.0\% \pm 2.3\%$ after 12 h and 24 h incubation respectively while in presence of nystatin, reduction in uptake was $65.4\% \pm 1.7\%$ and $37.8\% \pm 1.9\%$ after 12 h and 24 h respectively. Reduction in cellular uptake after 12 h and 24 h in presence of amiloride was found to be $50.2\% \pm 2.2\%$ and $22.2\% \pm 1.7\%$. From the results, it can be concluded that caveolae mediated endocytosis may be involved in the uptake of TNPs across the U87MG cell monolayer. The obtained results were also accordance with the reported literature (11)

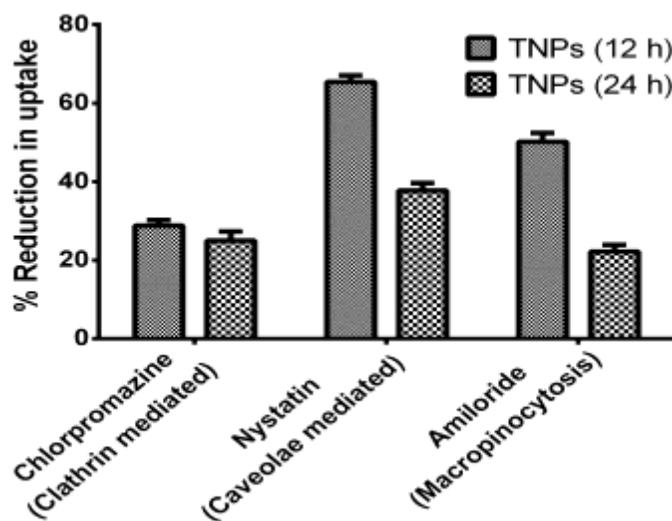


Figure 7.8: Elucidation of cellular uptake mechanism of TNPs on U-87 MG cells. Data is represented as mean \pm SD (n = 3)

In case of HA-TNPs, reduction in cellular uptake after 12 h and 24 h was found to be $32.6\% \pm 1.2\%$ and $25.2\% \pm 2.4\%$ (in presence of chlorpromazine), $46.3\% \pm 1.3\%$ and $55.3\% \pm 2.7\%$ (in presence of nystatin) and $41.7\% \pm 1.9\%$ and $17.6\% \pm 1.3\%$ (in presence of

Chapter 7 In vitro cell line studies of developed TNPs, HA-TNPs and CS-TNPs

amiloride) respectively (figure 7.9 A). From the results, it can be concluded that caveolae mediated endocytosis may be involved in the uptake of HA-TNPs across the U-87MG cell monolayer.

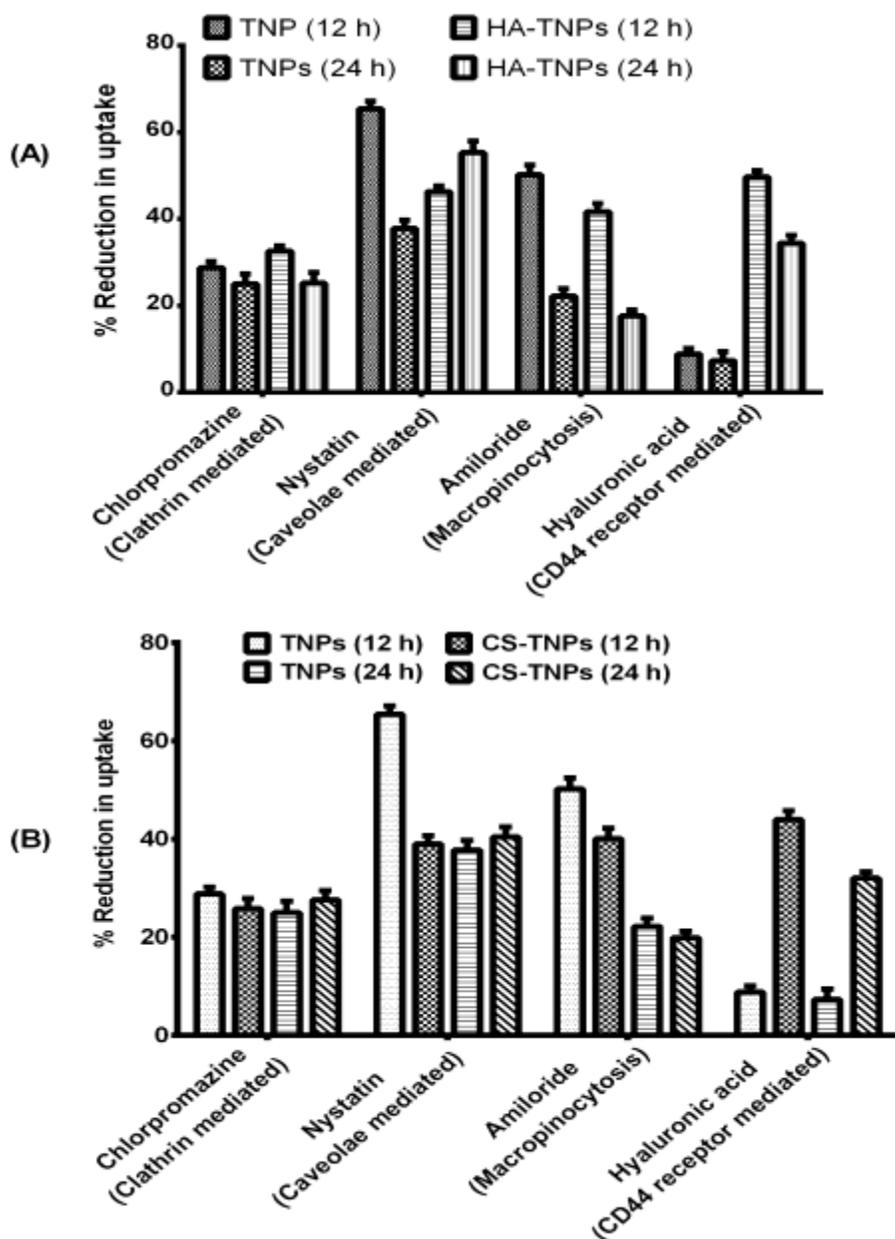


Figure 7.9: Elucidation of cellular uptake mechanism of (A) HA-TNPs and (B) CS-TNPs on U-87 MG cells. Data is represented as mean \pm SD (n = 3)

Chapter 7 In vitro cell line studies of developed TNPs, HA-TNPs and CS-TNPs

In case of CS-TNPs, reduction in cellular uptake after 12 h and 24 h incubation was found to be $25.8 \% \pm 1.4 \%$ and $27.6 \% \pm 2.3 \%$ respectively (in presence of chlorpromazine), $39.0 \% \pm 3.7 \%$ and $40.4 \% \pm 3.9 \%$ respectively (in presence of nystatin) and $40.1 \% \pm 3.2 \%$ and $19.9 \% \pm 1.7 \%$ respectively (in presence of amiloride) (figure 7.9B). From the results, it can be concluded that reduction in uptake was maximum when nystatin (caveolae pathway inhibitor) was used; so caveolae mediated endocytosis may also be involved in the uptake of CS-TNPs across the U-87 MG cell monolayer. The obtained results were also accordance with previously reported literature (12)

To verify the CD44 receptor mediated uptake of HA-TNPs and CS-TNPs, CD44 receptor blocking assay using excess amount of HA (10 mg/ml) as CD44 receptor inhibitor was used. The results of CD44 blocking assay indicated significant reduction in cellular uptake of HA-TNPs and CS-TNPs in HA treated U-87 MG cells as before CD44 receptor blocking, uptake was higher (figure 7.9). Free HA (as an inhibitor) hindered the binding of HA-TNPs and CS-TNPs with the receptor. The reduction in cellular uptake of HA-TNPs after 12 h and 24 h was found to be $49.7 \% \pm 1.4 \%$ and $34.4 \% \pm 1.8 \%$ respectively while in case of CS-TNPs, reduction in cellular uptake was found to be $44.0 \% \pm 1.4 \%$ and $32.0 \% \pm 1.8 \%$ respectively. These results confirmed CD44 mediated uptake of HA-TNPs and CS-TNPs in U-87 MG cells. In case of TNPs no significant reduction in uptake was observed which confirms that uptake of TNPs was not CD44 mediated. From all the obtained results, we can concluded that cellular uptake and internalization of HA-TNPs and CS-TNPs were predominantly via endocytosis and CD44 receptor mediated uptake.

7.4.6 ROS generation study

To assess the ROS generation potential of TNPs, HA-TNPs and CS-TNPs, we used DCF assay. Basically, H_2DCFDA is a non fluorescent dye and after entering the cells, it firstly gets hydrolyzed by the esterase into DCFH which was further oxidized into DCF in the presence of intracellular reactive oxygen species. This DCF is a fluorescent compound and its intensity of fluorescence is used as a marker for investigating the extent of oxidative stress (13). Based on this principle, the ROS generation potential of developed TNPs, HA-TNPs and CS-TNPs in U-87 MG cells was estimated and compared with the ROS of pure TMZ. The results as shown in

Chapter 7 In vitro cell line studies of developed TNPs, HA-TNPs and CS-TNPs

figure 7.10 and 7.11, demonstrated concentration dependent ROS generation of TNPs, HA-TNPs and CS-TNPs. The untreated cells (negative control) did not show any ROS generation while TMZ, TNPs, HA-TNPs and CS-TNPs demonstrated higher ROS generation as compared to positive control (H_2O_2). The TNPs, HA-TNPs and CS-TNPs indicated 2.57 folds, 3.86 folds and 4.55 folds increase in ROS generation respectively as compared to pure TMZ. The obtained results may be correlated with the fact that increase in concentration of TNPs, HA-TNPs and CS-TNPs led to increased concentration of released TMZ leading to increase in ROS generation. HA-TNPs and CS-TNPs showed higher ROS generation as compared to pure TMZ and TNPs. This may be due to higher uptake of HA-TNPs and CS-TNPs via HA/CS- mediated CD44 receptor which led to increased TMZ concentration in the cells that ultimately caused increased ROS generation that led to oxidative stress to the cells, inhibited the cell proliferation and caused cell death.

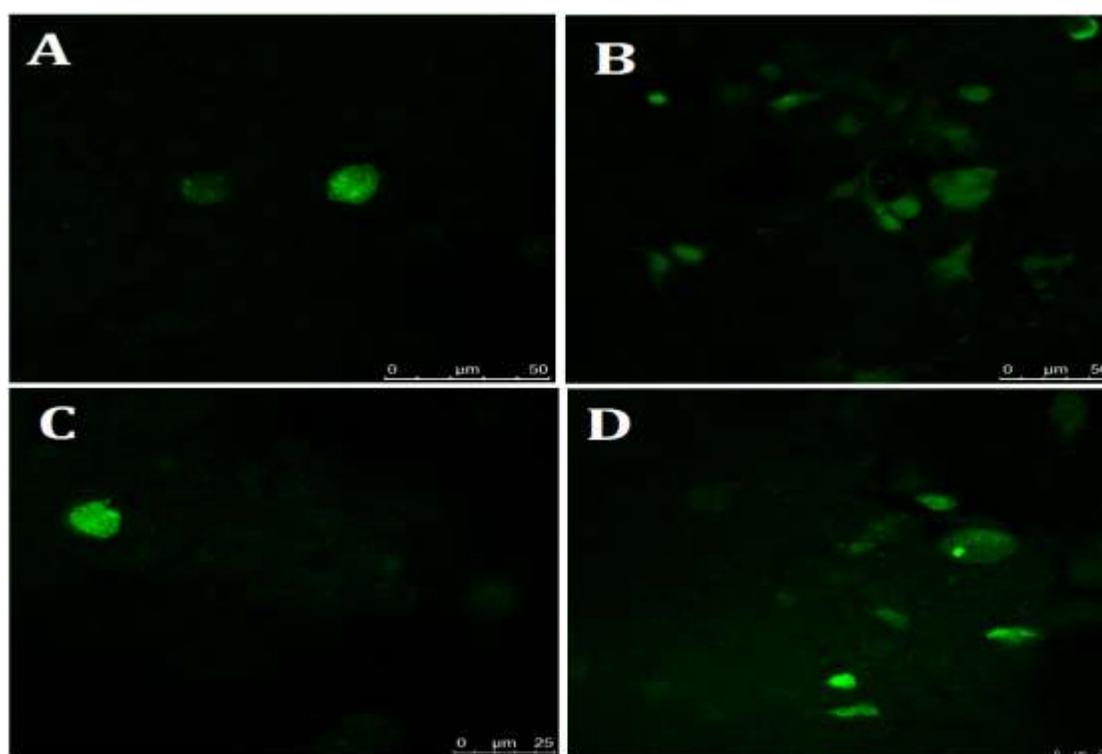


Figure 7.10: Qualitative determination of ROS generation (A) TMZ, (B) TNPs, (C) HA-TNPs and (D) CS-TNPs

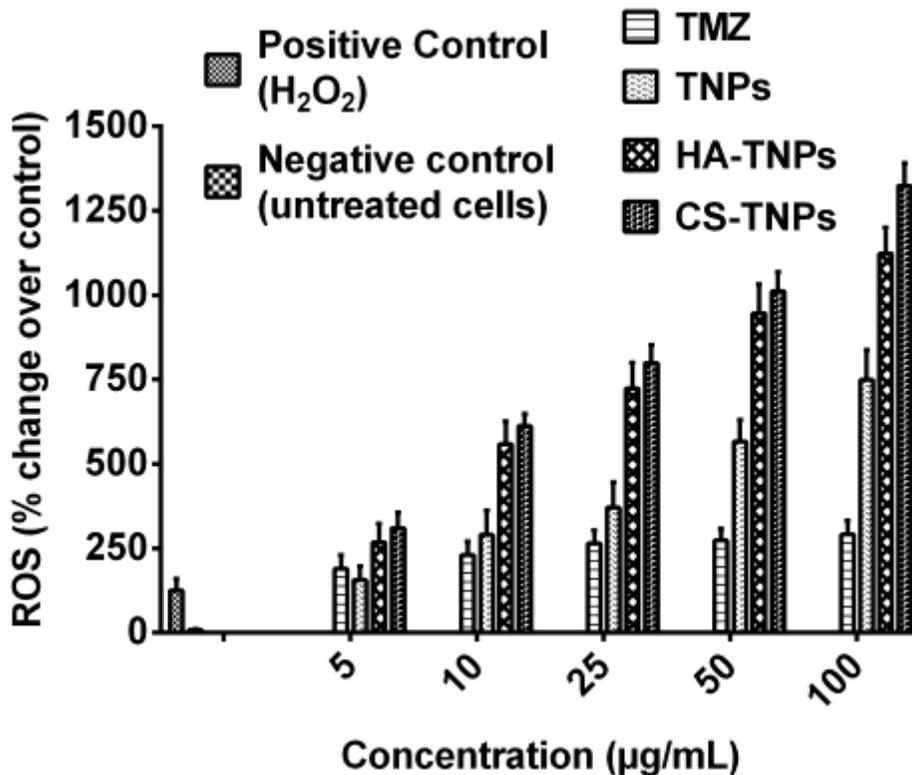


Figure 7.11: Quantitative determination of ROS generation (A) TMZ, (B) TNPs, (C) HA-TNPs and (D) CS-TNPs. Data represented as mean \pm SD

7.4.7 Scratch assay

The *in vitro* scratch assay was used to study cell migration as it is an easy, low-cost and well-developed method. The results of scratch assay are demonstrated in figure 7.12. The results indicated slower cell migration as compared to control group after treated with TMZ and developed nanoparticles. As compared to TMZ and TNPs, HA-TNPs and CS-TNPs indicated significant restriction of cell migration.

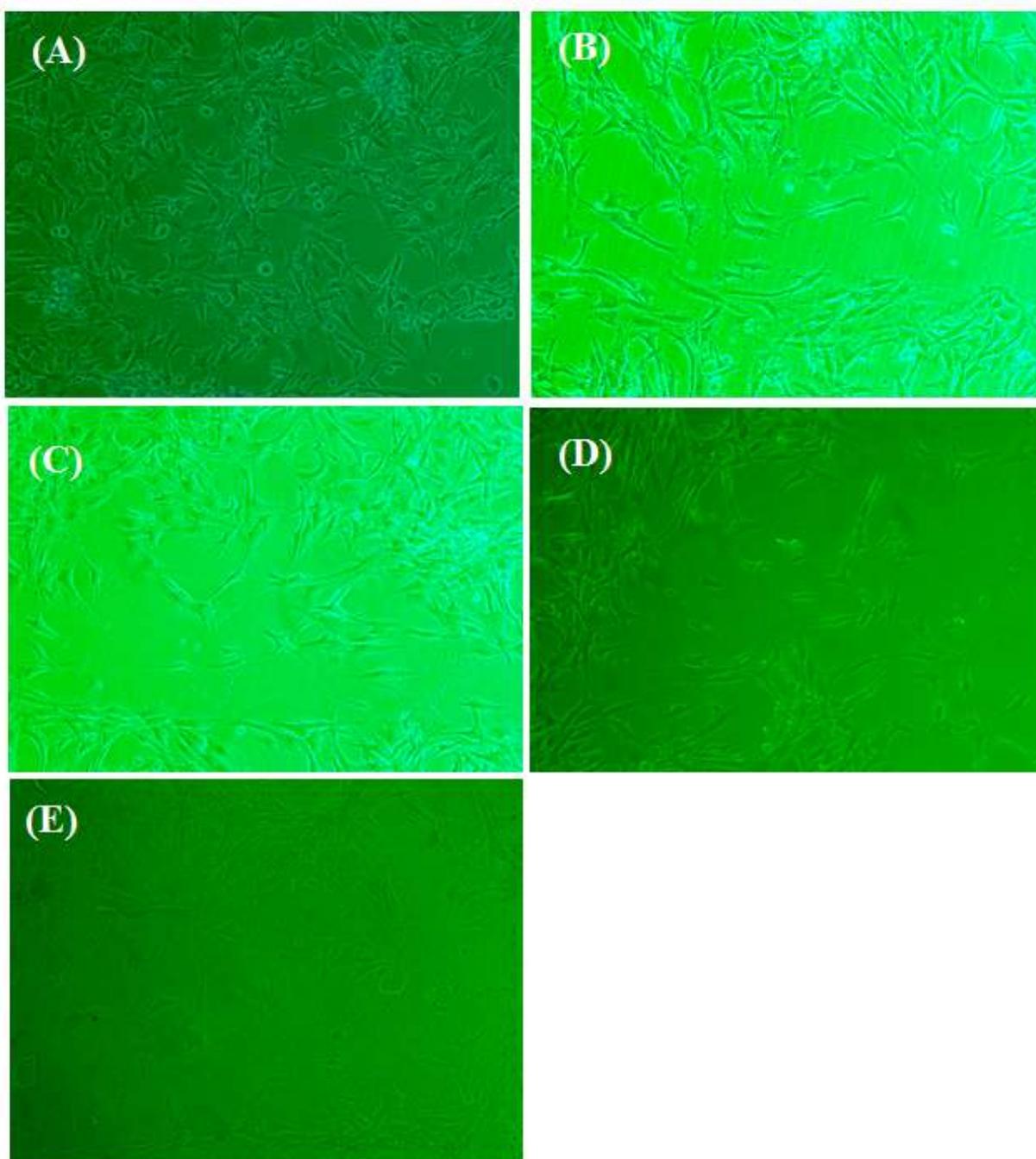


Figure 7.12: *In vitro* scratch assay to determine effect of different formulations on cell migration: (A) Control, (B) TMZ, (C) TNPs, (D) HA-TNPs and (E) CS-TNPs

Chapter 7 In vitro cell line studies of developed TNPs, HA-TNPs and CS-TNPs

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Chapter 7 In vitro cell line studies of developed TNPs, HA-TNPs and CS-TNPs

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