Original Article

Cytotoxic Assessment of Quinoline Based Derivatives on Liver Cancer Cell Line

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Abstract:

Current scenario of newer diseases with multiple causes has drawn the attention of the researchers in the field of therapeutics and they are now inclined to identify molecules effective for targeted therapy. **Objective:** Quinoline (1-azanaphthalene); belongs to heterocyclic aromatic nitrogen compound. Some quinoline-based derivatives are also known for their anti-tumor activity. The study was planned to evaluate the cytotoxic potential of quinoline derivatives. **Methods:** Berberine; a quinoline compound was made part of study to make structural analogs which were docked against potential target proteins. Cytotoxic profiling of all derivatives was done using MTT cytotoxicity assay. **Results:** The pharmacoinformatic and structure activity relationship studies of analogs were done. The cytotoxic profiles were elucidated by comparing viability rates of analogs treated hepatic cancerous cell line with untreated hepatic cells and untreated mesenchymal stem cells as standards. Marked cytotoxicity was seen in all molecules at low doses than reported in past studies with relevance to parent compound. **Conclusions:** The results will be further confirmed through various other cell culture assays targeting different marker proteins, pharmacoinformatics tools and structure activity relationship studies.

Key words: Quinoline, Berberine, Cytotoxicity, Mesenchymal stem cells, Cell viability

Introduction:

Isoquinoline; structural isomer of quinoline, are heterocyclic aromatic organic compounds with hygroscopic nature and an unpleasant odor. Isoquinoline is a benzopyridine and is employed in the manufacturing of paints, dyes, insecticides and also as a solvent for extraction of terpenes and resins. Derivatives of isoquinolines compounds are widely renowned for their extensive application as anesthetic such as dimethisoquin; antihypertensive such as debrisoquine, quinapril; vasodilator such as papaverine, besides antifungal and disinfectant properties [1].

Berberine belongs to quinoline groups which are widely studied molecules to date. The wider spectrum of quinolines's activities may be attributed to their synthetic flexibility which offers an extensive range of structurally sundry products. These various molecules are made through substitution and derivatization in the ring system of quinolines.

Berberine is an isoquinoline alkaloid which is extracted from *Berberis vulgaris* and has been promisingly reported for its anti-proliferative effects on tumor cells and targets multiple pathways. The anti-proliferative and apoptotic effects of Berberine were checked by Lin SZ et al on HL-60 cells and tendency of vascular endothelial growth factor and receptor-2 was analyzed. At doses of 6-96 µg/ml anti proliferative effects were estimated and mRNA was checked by using Real time PCR and western blotting techniques. Berberine appeared to reduce the proliferation in a concentration dependent mode. The cells were markedly decreased in S- phase [2-6].

PQ7; a second generation substituted quinoline was synthesized to increase the gap-junctional

intra-cellular communication (GJIC), which effectively retards growth of cancer cells. The results were tested against T47D breast cancer cell line [7, 8, 9, 10, 11].

Berberine effects were seen on epidermoid carcinoma cell line; A431 in a concentration range of 0-75 µM through MTT assay and marked inhibitory results were observed [12]. Similar study was done by Mantena SK in the same year but that time using cell lines DU145, PC-3 and LNCaP, and, G-1 phase cell cycle arrest was observed by berberine in human prostate cancer cells by employing MTT assay at a concentration of 0-100 µM. Berberine was applied at a concentration range of 0-100 µM on RMPI-8226 cell line, and, suppressing effects were seen on human myeloma cells after MTT assay [13]. On cell line SMMC-7721, at a concentration range of 0-89 µM doses of berberine, MTT test was done for effects in hepatocellular carcinoma and proficient effects were the result [14]. Cell lines SC-MI, CL1-5 were employed to check HIF-1 alpha expression by berberine through MTT assay at dose range of 0-100 µM [15].

In a study on vascular smooth muscles; 0-300 µM was the concentration range at which berberine was applied on VSMC cell line and inhibition of proliferation was seen on vascular smooth muscles using MTT technique [16]. A study done on human glioblastoma cells by using T98G cell line was conducted, and, marked apoptosis and G1 arrest occurred through MTT assay [17]. For identification of NF-kb involving mechanistic pathway of berberine, marked suppression in antiapoptotic gene products was observed after applying MTT assay [18]. Another study done to elucidate mechanism of apoptosis by berberine through microRAN-21 and nuclear factor kappa b (NF-kB) pathways, was conducted in 2013 and the assessment assay was MTT [19, 20].

Methods:

Chemicals and Reagents

All the reagents and chemicals employed in this study were of analytical grades and mostly were acquired from Merck Chemical Co. and Sigma; Aldrich. Berberine; an alkaloid extracted from various plants, is potentially exploited for its wider spectrum of reported uses, was used as parent compound for the synthesis of derivatives and various combinations [21-25]. Structural derivatization was done through a series of chemical reactions, scheme of which along with their structure activity relationship studies will be shared in further publication.

Materials

Dulbecco modified eagle medium (DMEM), L-glutamine 200 mM, Penstrep 10% fetal bovine serum (FBS), versene-EDTA and phosphate buffer saline (PBS) were purchased from GIBCO Invitrogen, USA. Methyltetrazolium salt (MTT) and DMSO were obtained from MB Cell, Korea. Sodium dodecyl sulphate (SDS) was purchased from Vivantis, USA. 96 well tissue culture plates were acquired from Orange Scientific, USA. Tissue culture flasks and 6 well culture plates were the product of Oxygen Life Sciences, California.

Sampling

Sampling of UC-MSCs

The large quantity of umbilical cord transforms into billions of stem cells which are cultivated in primary cultures. Those cultures are utilized in clinical research settings and go advantageous as they are in-expensive and proliferate at a good rate [26-29].

In our study we used Umbilical cord mesenchymal stem cells (UCMSCs) as another control along with untreated human liver cancer cell line for comparing rates of proliferation. UC-MSCs were collected from Services Hospital, Lahore. Strict criteria were made to include mothers who were free from HIV, Hepatitis C and Hepatitis B after filling and signing informed consent. The sterile container with normal saline was added with 100 U of penicillin (GIBCO, USA) and 100 µg/ml streptomycin (GIBCO, USA).

Sampling of Hep-GCs

Human liver carcinoma cells (HepG2) is a wholesome cell line that is frequently employed as HCC for clinical researches. Many biotransformation studies utilize hepatoma cell

lines and primary hepatocytes as in *vitro* model, whereas the role and use of these cell lines to study and understand xenobiotics metabolism, liver toxicities, cytotoxic and genotoxic analyses, drug targeting and hepato-carcinogenesis, is imperative [30, 31]. Since 1980, Hep G2 has been listed as a human cell line (HB 8065) on the American Type Culture Collection (ATCC, Rockville, MD, USA) repository. Hep G cell line was attained from the cell and tissue culture laboratory of Centre for Research in Molecular Medicine (CRiMM) established in the University of Lahore (UOL). Cell line was saved and stored in liquid nitrogen and contained in-cryo vials which were invigorated for additional handling.

Isolation and culturing

Isolation and culturing of UC-MSCs

Cord was taken out from the bottle and wash-down with normal saline solution to eliminate the contamination, this way contamination from its umbilical veins was also removed. After washing and removing contamination the cord was placed on a petri-plate and cut down into pieces. Usual size of the cut pieces of cord was 1-2 inches. Moreover, the minced pieces, that acts as explants were plated on to the culturing flask with the help of forceps. The medium was added in the culturing flask containing minced cord pieces. Dulbecco Modified Eagle Medium high glucose (DMEM HG) was used for culturing. In this medium 10 % platelet rich plasma (PRP) and 100 U of penicillin and 100 µg/ml of streptomycin were added. The medium was changed from the culturing flask after 3 days. When culture of UC reached 70-80 % confluence their sub-culturing was done. Culture flask was washed with normal saline solution. After the addition of trypsin-EDTA culture flask was incubated for 2-3 minutes so that cells could detach. After detachment medium PRP was added to stop effect of trypsin. It was then transferred to 15 ml falcon tube and centrifugation was done. Pellet was re-suspended in medium and sub-cultured into another flask.

Culturing of Hep-GCs

The cryo vials were taken out from liquid nitrogen cylinder and thawing was done. The Hep G cell line was grown in culturing flasks which contained DMEM-HG, PRP, Streptomycin and Penicillin. When 70-80% of confluence was achieved by cultivated Hep-G cells, then further splitting was done. For the step of splitting, the cells which were attached with the walls of the culturing flask were given a wash with normal saline and incubation was provided with trypsin-EDTA until detachment of cells from the walls was observed. Detachment was confirmed through microscopic examination of the flasks. Addition of PRP was done with constant stirring and centrifugation of whole mixture was carried out in a 15 mL tube for 5 minutes at 2000 rpm. After this the supernatant was detached and pellet was re-platted.

Treatment of Hep-GCs and UC-MSCs with derivatives

Hep-GCs and UC-MSCs at second passage were cultured onto 96-well plates for cell proliferation assay. Cultured Hep-GCs and UC-MSCs. A total of eight (8) study groups were made and named as; UT-MSCs; Control I (untreated mesenchymal stem cells), UT-HGCs; Control II (untreated hepatic cancer cells), T-BBR; Control III (compound BBR treated Hep-GCs), T-BBR-4, T-DP-1, T-BBR-NA, T-BBR-ANS, T-K3-GA-DEA and, T-K3-GA-BBR. Treatment was given to cultured cells for 24 hours. After 24 hours, 96 well plates were labeled for cell proliferations assay and the harvested medium of all experimental groups was used for further biochemical assays.

Cell proliferation Assay

Enzyme-based colorimetric method uses MTT salt as a reducing coloring agent for the dehydrogenase enzyme that is expressed by viable cells. High reproducibility, easy-to-employ and safety are the associative features of this assay for which it is practiced commonly and is far superior on other available methods. Among all the assays designed up till now to measure the mitochondrial dehydrogenase activities of living cells, MTT method is best

studied and well-known. In the assay, reduction of MTT takes place by NADH to formazan; an insoluble needle shaped and purple colored crystalline compound. Insoluble crystals are needed to solubilize by organic solvents, prior to measure absorbance [32, 33].

For comparison of proliferative potential of experimental groups on Hep-GCs, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was conducted on cultured cells in 96 well plates. Eight different concentrations i.e., 4μM, 8 μM, 16 μM, 32 μM, 64 μM, 100 μM, 200 μM and 400 μM were applied on cells in triplicate and whole assay was repeated for three times. Monolayer of cells was given first washing with phosphate buffer saline (PBS) (Invitrogen Inc., USA) followed by incubation for 2 hours in 500 μL medium containing 60 μL of MTT solution (Invitrogen Inc., USA). MTT formed insoluble purple colored formazan crystals in live cells. 10% sodium dodecyl sulphate (SDS) was used to solubilize those insoluble crystals of formazan before measuring absorbance which was taken at 570 nm.

Percentages of viable and dead cells were estimated using following formula:

$$\%\text{viable cells} = \frac{\text{Abs. of sample}}{\text{Abs. of control}} \times 100$$

Results:

A total of six molecules were synthesized and assigned codes as BBR-4, DP-1, BBR-NA, BBR-ANS, K3-GA-DEA and K3-GA-BBR. Treatments were given to hepatoma cell line with all compounds at concentration of 4 μM, 8 μM, 16 μM, 32 μM, 64 μM, 100 μM, 200 μM and 400 μM respetively. Untreated mesenchymal stem cell (UT-MSCs) and untreated hepatoma cell line (UT-HGCs) were taken as control. Figures 1-8 show the graphs which were plotted taking concentrations on x-axis and percentage viable/ dead cells on y-axis.

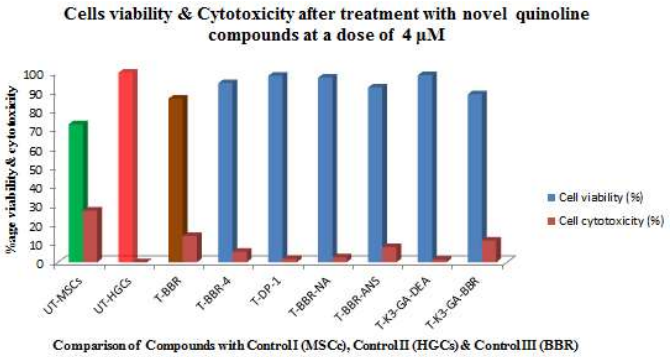

Figure 1: Graphical represetation of viable and dead cells after treatment of hepatoma cell line with quinoline compounds at a dose of 4 μM

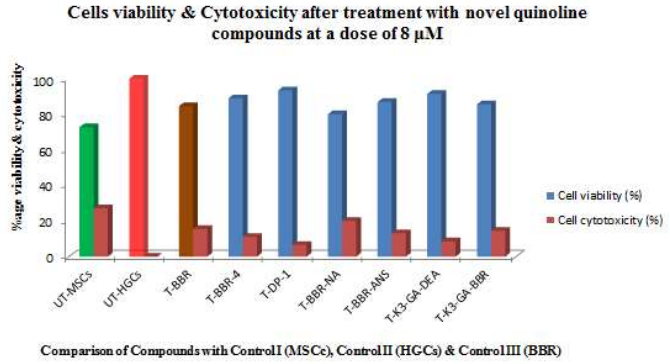

Figure 2: Graphical represetation of viable and dead cells after treatment of hepatoma cell line with quinoline compounds at a dose of 8 μM

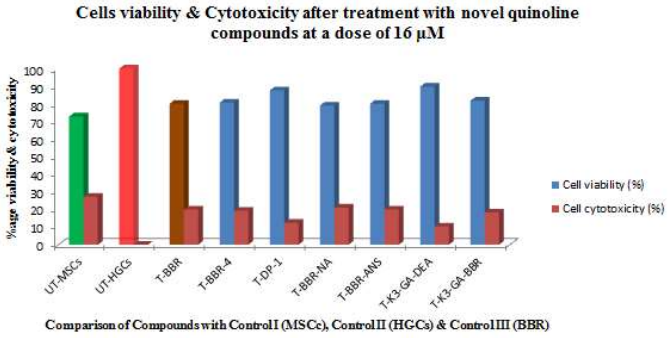

Figure 3: Graphical represetation of viable and dead cells after treatment of hepatoma cell line with quinoline compounds at a dose of 16 μM

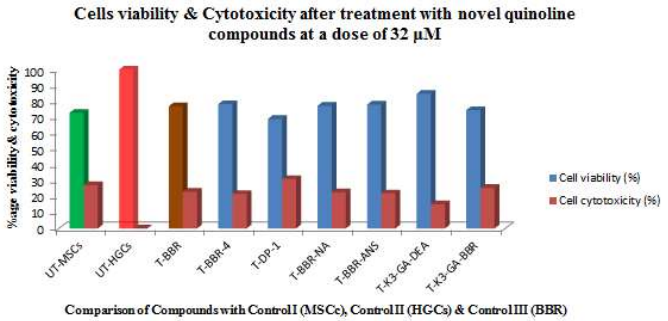

Figure 4: Graphical represetation of viable and dead cells after treatment of hepatoma cell line with quinoline compounds at a dose of 32 µM

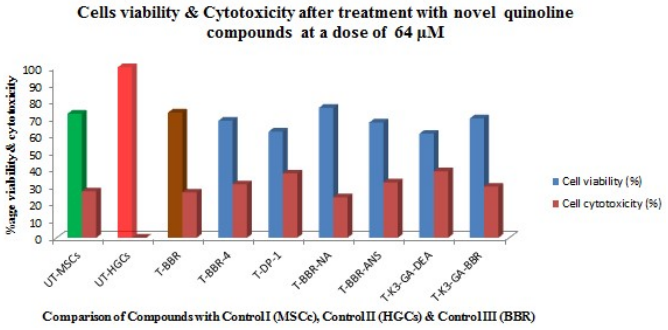

Figure 5: Graphical represetation of viable and dead cells after treatment of hepatoma cell line with quinoline compounds at a dose of 64 µM

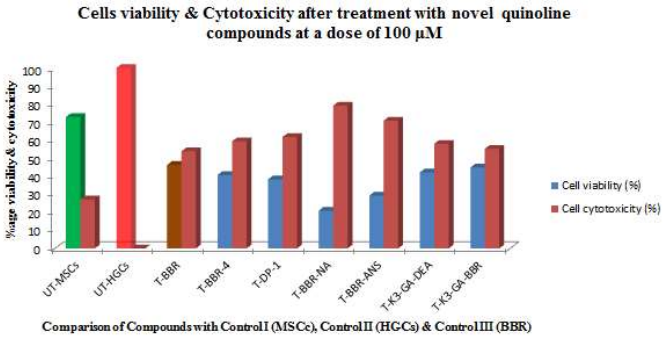

Figure 6: Graphical represetation of viable and dead cells after treatment of hepatoma cell line with quinoline compounds at a dose of 100 µM

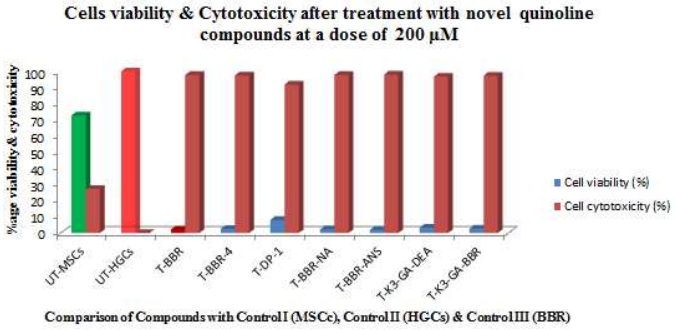

Figure 7: Graphical represetation of viable and dead cells after treatment of hepatoma cell line with quinoline compounds at a dose of 200 µM

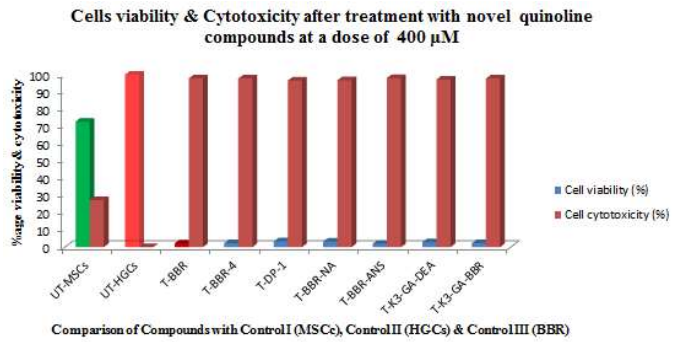

Figure 8: Graphical represetation of viable and dead cells after treatment of hepatoma cell line with quinoline compounds at a dose of 400 µM

Discussion:

Ample researches have been done on phytochemicals to identify propitious results in the field of cancer [34]. Berberine, one such bioactive compound is isolated from *Berberis vulgaris* has got publicity for its wider pattern of effects owing to its mechanisms at cellular and molecular level [35].

Many biotransformation studies utilize hepatoma cell lines and primary hepatocytes as in *vitro* model, whereas the role and use of these cell lines is imperative to study and understand xenobiotics metabolism, liver toxicities, cytotoxic and genotoxic analyses, drug targeting and hepato-carcinogenesis, is imperative [31]. The large quantity of umbilical cord, that connects placenta and fetus during pregnancy, transforms into billions of stem cells which are cultivated in primary cultures. Those cultures

are utilized in clinical research settings and go advantageous as they are in-expensive and proliferate at a good rate [27-29]. Both human liver carcinoma cell line and umbilical cord mesenchymal stem cells were employed in our study. The drug treated liver cancerous cells were compared with proliferative results of controls; untreated human liver carcinoma cells and untreated mesenchymal stem cells.

Proliferative potential of cells treated with the compounds was estimated through MTT assay. High reproducibility, easy-to-employ and safety of this assay compelled us to employ this in our study [33]. Reduction of MTT took place by NADH to formazan; an insoluble needle shaped and purple colored crystalline compound. Insoluble crystals were solubilized by organic solvents, prior to measure absorbance. The absorbance of those cells was less which were less proliferative by the use of drug. Eight different concentrations; 4µM, 8 µM, 16 µM, 32 µM, 64 µM, 100 µM, 200 µM and 400 µM; were used on hepatoma cell line, and, an indirect relation was noted between dose and absorbance i.e., higher the dose, lesser was the proliferation and so was the absorption which was taken at 570 nm.

Graphs were plotted between absorbance noted during MTT assay after applying eight different concentrations; 4 µM, 8 µM, 16 µM, 32 µM, 64 µM, 100 µM, 200 µM and 400 µM of drugs. The absorbance of control i.e., proliferative un-treated stem cells and proliferative un-treated hepatoma cells were 2.110 and 2.900 respectively. Minimum inhibitory concentrations were best considered at dose of 64 µM where parent compound BBR showed absorbance 2.130 and least proliferations were shown by all drugs. All results were found significant in case of BBR-ANS treated cells when compared with those un-treated cells. Percentages of the viable cells were calculated by dividing absorbance of sample with control and then multiplying the value with 100 and graphs were plotted between cell viability and cytotoxicity given by each concentration for every compound along with control for comparison.

We see many past researched done on berberine which clearly indicate the potent anti-cancerous role of berberine through many mechanisms. Berberine suppresses cancer cells via regulating cell cycle, inhibiting the ATP generation, or inducing cancer cell apoptosis [35, 36]. Berberine has also been tested for controlling oncogene at transcriptional level and, a well-documented data supports such inference. Inhibitory results of berberine have been validated for many microorganisms that induce and propagate tumors [37]. When we compare our results with the studies done in past then, proficient results of our novel compounds through MTT assay in low doses on Hep-G cell line also show them promisingly anti-proliferative.

Conclusions:

The cytotoxic pattern was potentially demonstrated by quinoline compounds on hepatoma cell lines in low doses. The inhibitory mechanism of derivatives on tumor induced angiogenesis was elucidated when compared with mesenchymal stem cells as standard control on hepatic carcinoma cell line (HCC). Profound effect was seen by all compounds in inducing cellular toxicity in cancerous cell line. Promising results are compelling enough to be validated on various cancerous cell lines and also their structure activity relationship studies will be further done through high throughput techniques.

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