

In vitro single-strand DNA damage and cancer cell cytotoxicity effects of Temozolomide

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Abstract

Temozolomide (TMZ) is approved against certain tumors, but substantial evidences are scarce to demonstrate TMZ as single-strand DNA break agent and reasons behind lack of responses in carcinoma. Plasmid and genomic DNA included as a substrate during assessment of *in vitro* single-strand break potential of TMZ. Cell based assay was performed to assess DNA damaging activity and cytotoxicity in HeLa. Further, authors tried to evaluate DNA damage and overall cytotoxicity in HeLa using Trypan blue dye exclusion and apoptotic DNA fragmentation assays. Data indicate that TMZ can generate extensive single-strand break on different DNA substrate. A DNA ligase inhibitor L189 appears to plays synergistic role in TMZ ability to decrease cell proliferation in HeLa and some extent of cytotoxicity. However, authors noticed none apoptotic DNA fragments due to toxicity effects of TMZ alone or combined TMZ plus L189. Based on our observations, findings support combined use of TMZ and L189 inhibitor to enhance responsiveness in HeLa possibly by decreasing cellular growth instead of apoptotic cell death pathway.

Key words: Nicking, Genotoxic drugs, Cancer, Supercoiling, DNA relaxation, Temozolomide.

Introduction

In recent, rapid emergence of cervical cancer is associated to higher cancer burden in women. Therefore, these diseases are of major societal and familial consequences [1, 2]. Over several decades, many therapies are standardized to control cancer growth and proliferation like chemotherapy and radiation therapies due to their genotoxic effect [3, 4]. Many anticancer drugs act by creating genomic disturbances and instability leading to cell cytotoxicity [3-6]. Chemotherapeutic agents like alkylating agents, heavy metals antimetabolites and cytotoxic antibiotics and DNA topoisomerase inhibitors are known to cause DNA damage like single or double strand break [7-10].

Temozolomide (TMZ), an imidazotetrazine is converted to a compound capable of alkylating DNA. TMZ does that by non-enzymatically converting to MTIC (5-3-(methyltriazen-1-yl) imidazole-4-carboximide) at physiologic pH and degrades to methyl diazonium cation. Thereby delivering a methyl group to purine bases in the DNA and thus stalling DNA replication and leading to cytotoxicity in proliferating cells [7, 11-15]. There is evidence that methylation action by TMZ could be noticed at the N⁷ position of guanine, O³ position of adenine and the O⁶ position of guanine [16-19]. In certain carcinoma, there is an existence of correlation between the sensitivity of tumor cell lines to TMZ and activity of DNA repair proteins such as Methyl-guanine methyl

transferases and mismatch repair (MMR). At the same time contribution of Base Excision Repair (BER) protein is also warranted for cancer drug responsiveness [16-19].

However, it is also suggested that TMZ does not produce direct (frank) single strand breaks, they are observed in cells as incomplete BER, and *in vitro* as a product of slow DNA hydrolysis due to alkylation causing a weakening of the glycosidic bond [7, 20-26]. In BER pathway, DNA ligases are necessary to seal the gaps or single-stranded damage occurred in DNA. Recently, efforts are noticed to inhibit DNA ligases by blocking the DNA repair machinery to create cytotoxic environment in carcinoma [24, 27, 28]. Based on existing knowledge of TMZ in several cancer types for possible drug resistance, authors find lack of evidence to demonstrate single strand DNA cleavage activity of TMZ and responsiveness in HeLa.

In this article, authors attempt to provide experimental evidences to demonstrate nicking and DNA damage activity of TMZ during *in vitro* DNA substrate break assay. Further, authors show the response of TMZ in HeLa with or without DNA ligases inhibitor L189.

Materials & Methods

Drugs and Reagents

Drug TMZ purchased from Cayman Chemical Ltd. DNA Ligase Inhibitor (L189) was purchased from Santa Cruz Biotechnology. Reagents as DMSO, H₂O₂, TAE, Proteinase K, RNase, Ethidium bromide, Trypsin, PBS, DMEM, high glucose, DPBS, Propidium Iodide, NFW (Nuclease Free Water), pBR322 plasmid DNA and pUC18 plasmid DNA were of molecular biology grade and procured from HiMedia Laboratories (Mumbai, India) and Merck. The cancer cell lines HeLa was obtained from National Center for cell Science, Pune.

Cell Line Maintenance

The HeLa was grown in DMEM (Dulbecco's Modified Eagles Medium) with 10% FBS (Fetal Bovine Serum) and Antibiotic solution containing 10000 units of Penicillin and 10 mg Streptomycin in humidified CO₂ incubator with 5.0% CO₂ level.

Genomic DNA Isolation from bacterial and animal cells

Genomic DNA isolation was performed by phenol-chloroform extraction method. The overnight grown culture of DH5a was subjected to lysis and protein denaturation by SDS and proteinase, respectively. Further removal and denaturation of

protein contaminants was carried out by phenol: chloroform method. Sodium acetate was used for nucleic acid precipitation there after resuspending in TE buffer and treating it with RNase. Extracted DNA was quantified using spectrophotometric method.

Plasmid and genomic DNA Nicking Assay

Five μ L of pBR322/pUC18 plasmid DNA (50 μ g/ml) and one μ g of genomic DNA were mixed with 2 μ L each of TAE buffer (Tri-acetate/EDTA 10 mM, pH 7.4). Further, reaction mixtures were added with different reaction substrates like H₂O₂ (5 mM to 500 μ M), DMSO (0.5 mM to 2.5 mM), and TMZ (0.5 mM to 2.5 mM) for these assays, cautions were taken to maintain the same solvent percentage in each mixture. Final volume of each reaction mixture was brought to 25 μ L by addition of nuclease free water in a microcentrifuge tube. Reaction mixtures were respectively incubated for 24 h at 37°C. Following incubation, 2 μ L of loading dye was added to the incubated mixture, and 10 μ L was loaded onto a 1% (*w/v*) agarose gel. Electrophoresis was conducted at 100 volts in Tris-Acetate-EDTA • Na₂ (TAE) buffer (0.04 M tris-acetate and 1 mM EDTA, pH 7.4) using a Horizon 58 (Life Technologies, Waltham, MA, USA). The agarose gel was stained with ethidium bromide for 15 min. DNA bands were visualized with a Bio-Rad Laboratories India Pvt. Ltd (Gurgaon, Haryana, India) Gel Doc™ EZ imager.

Quantification of nicked, linearized and damaged DNA

In order to quantify single-strand nicking, plasmid DNA form supercoiled (Form I), nicked (Form II) and linearized bands (Form III) were visualized using Bio-Rad Gel Doc™ EZ imager and their intensities quantified with the help of ImageJ software, Bethesda, Maryland, USA.

Determination of cell viability assessment by trypan blue dye exclusion assay

HeLa was trypsinized from 75cm² culture flask and were centrifuged to form a uniform cell suspension. For the total cell count 10 μ L of suspended cells were observed on a hemocytometer. For viability checking 10 μ L of cell suspension was mixed in equal amount of filter sterilized trypan blue dye and cell was counted as per routine procedure.

Apoptotic DNA isolation

The harvested cell pellet was washed with PBS and centrifuged further by suspending in lysis buffer for 10 sec. Then, it was centrifuged again at 1600 X g for 5 min and repeated the lysis step. After

centrifugation, supernatant was collected and treated with RNase for 2 h at 56°C (final concentration 2.5 µg / µL). In the next step, samples were digested with Proteinase K for 2 h at 37°C (final concentration 2.5 µg/µL). Further, 1/2 volume of ammonium acetate was added and precipitated with 2.5 volume of ethanol. The precipitated DNA was resuspended in TE buffer. Lastly, DNA was quantified [29].

Statistical analysis

The experiments were conducted independently three times. Results are expressed as mean ± SD. Data from different assays were statistically compared using t-test. Statistical significance was acceptable to a level of $p < 0.05$.

Results

Effect of TMZ concentration on plasmid DNA nicking

To assess DNA single strand nicking activity of TMZ, plasmid DNA substrate is commonly used as substrate. Usually, there are three different forms of plasmid DNA as supercoiled DNA, relaxed DNA and linearized DNA. Lower band in the control lane represents intact supercoiled circular DNA. Upper band in the rest of the lanes represents relaxed and cleaved DNA. Plasmid DNA conformation is reported to change due to the action of oxidants or genotoxic

drugs. Ethidium bromide stained gel photograph is presented in Figure 1(A) and (B). DNA damage and nicking are evaluated at TMZ concentration of 0.3 mM, 0.5 mM, 1 mM and 2.5 mM. The maximum relaxation of the plasmid DNA was noticed at 1 mM TMZ. Data depict that after increasing concentration up to 2.5 mM, linearized DNA form is observed as shown in Figure. 1(C). DNA nicking data indicates 96% of relaxation caused because of single-strand DNA nicking at the concentration of 1 mM. Hence, TMZ at 1mM is considered optimum for further single-strand break DNA damage analysis and cell cytotoxicity assay.

Plasmid DNA nicking of TMZ at different time points

To determine optimum time in nicking assay, 1 mM of TMZ was tested for 6 h, 12 h and 24 h. The results are presented as ethidium bromide stained gel photograph and their quantitative density analysis. Nicking reaction confirmed that activity of TMZ is maximum at 24 h as shown in Figure 2.1 (A) & (B). At the same time, at 12 h and 6 h duration, plasmid DNA seemed to be nicked less as depicted in Figure (2.2 (A) & (B) and Figure 2.3 (A) & (B), respectively. During 24 h of drug treatment, data suggest that maximum supercoiled DNA was converted into relaxed form.

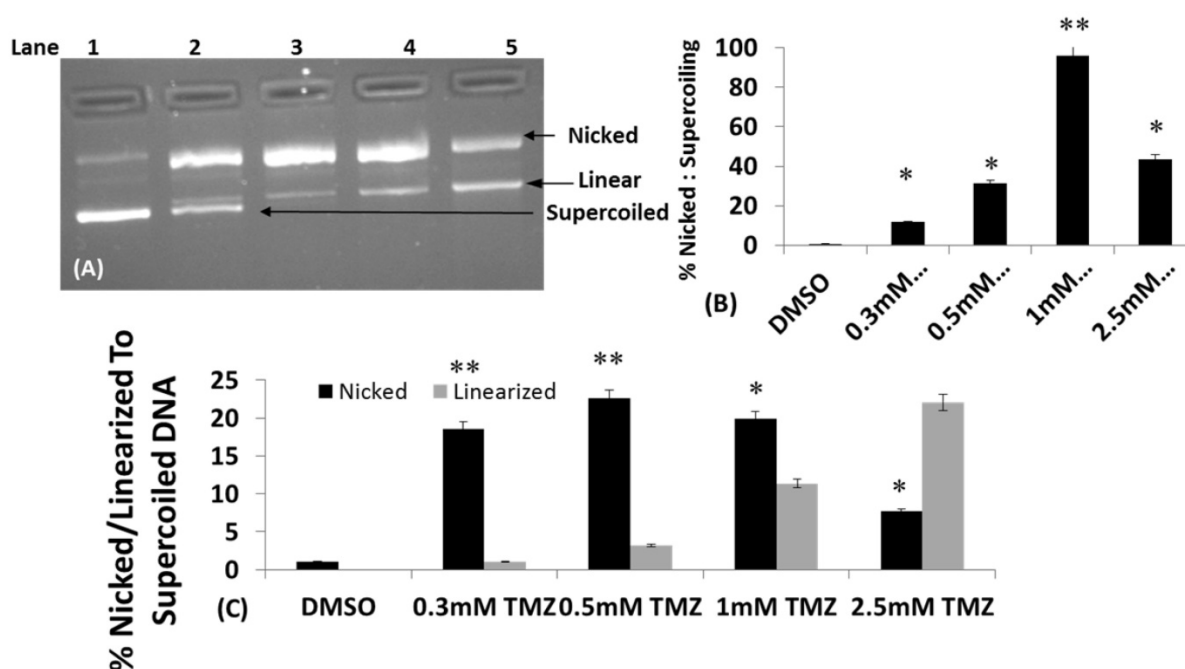


Figure 1. Effect of Different TMZ Concentration as DNA Single-Strand Break: (A & B) Different concentration of TMZ (Lane1-DMSO Control; Lane 2- 0.3 mM TMZ; Lane 3- 0.5 mM TMZ; Lane4- 1 mM TMZ; Lane 5- 2.5 mM) Maximum nicking of plasmid DNA was observed at 1mM when compared to DMSO control, further increase in TMZ concentration led to linearization of the DNA (C) Amount of nicked and linearized DNA with increasing TMZ concentration. The data are represented as mean ± SD. Each experiment was conducted independently three times. * Significantly different from DMSO control at P-value < 0.05. ** Significantly different from DMSO control at P-value < 0.01.

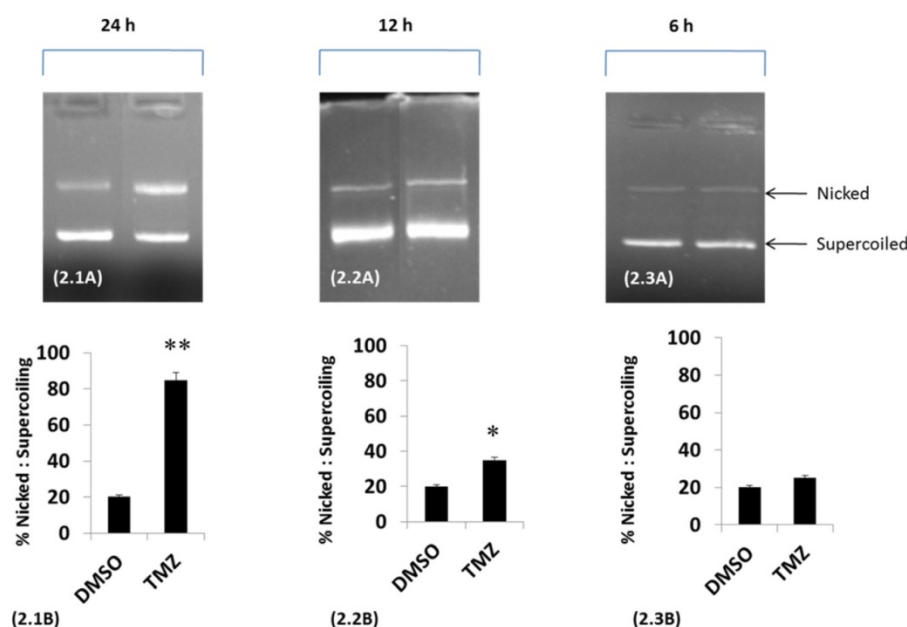


Figure 2. Plasmid DNA nicking by TMZ at different time points at 24 h, 12 h and 6 h as shown in (2.1 A & B) (2.2 A & B) (2.3 A & B) respectively (Lane 1-DMSO control; Lane 2- TMZ (1mM)). Action of TMZ at 6hrs and 12 h had no effect on plasmid DNA whereas at 24 h nicked Plasmid DNA was observed. The data are represented as mean \pm SD. Each experiment was conducted independently three times. * Significantly different from DMSO control at P-value < 0.05. ** Significantly different from DMSO control at P-value < 0.01.

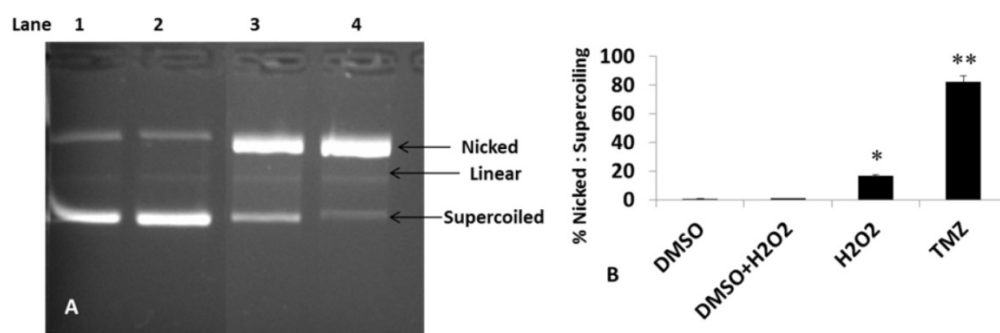


Figure 3. Action of DMSO on nicking of the plasmid DNA. Figure. 3A & 3B. When treated DMSO no conformation change in DNA was seen (Lane 1), H₂O₂ which was taken as positive control, but combinatorial effect of DMSO and H₂O₂ resulted in quenching the damaging effect of H₂O₂ (Lane 2) whereas TMZ was potent DNA damaging agent (Lane 4) (Lane1-DMSO Control; Lane 2-DMSO (1 mM) + H₂O₂ (500 μ M); Lane 3- H₂O₂ (500 μ M); Lane4- 1mM TMZ). The data are represented as mean \pm SD. Each experiment was conducted independently three times. * Significantly different from DMSO control at P-value < 0.05. ** Significantly different from DMSO control at P-value < 0.01.

Effect of DMSO against nicking activity of TMZ

To justify TMZ as single-strand break agent, reaction is carried out with H₂O₂ as a positive control due to its known mechanism to inflict DNA damage. The gene analysis marks that TMZ at 1 mM produce better single-strand relaxation activity compared to H₂O₂ (500 μ M). Combination of DMSO and H₂O₂ shows the quenching effect of DMSO upon H₂O₂ as presented in Figure 3. (A) & (B). Anti-oxidant nature of DMSO prevents free radicals H₂O₂ caused DNA nicking. It is also observed that DMSO as a solvent did not affect nicking activity of TMZ.

DNA nicking against bacterial and animal cell genomic DNA

In view of extending types of DNA substrate, authors included bacterial and Hela genomic DNA to corroborate DNA damage effects as shown in plasmid DNA. Data suggest that TMZ not only acted upon plasmid DNA due to unique DNA sequence, but also extensively degraded the bacterial and animal genomic DNA (Figure 4A & B, Figure 4C & D). As the quantitation of genomic DNA nicking was done, result reports up to 80% genomic DNA degradation in both bacterial as well as animal genomic DNA.

Therefore, the results indicate that the potency of TMZ as a single-strand DNA damaging agent is working against both plasmid and genomic DNA

substrate. In this data, H₂O₂ was used a positive control and DMSO as normal control.

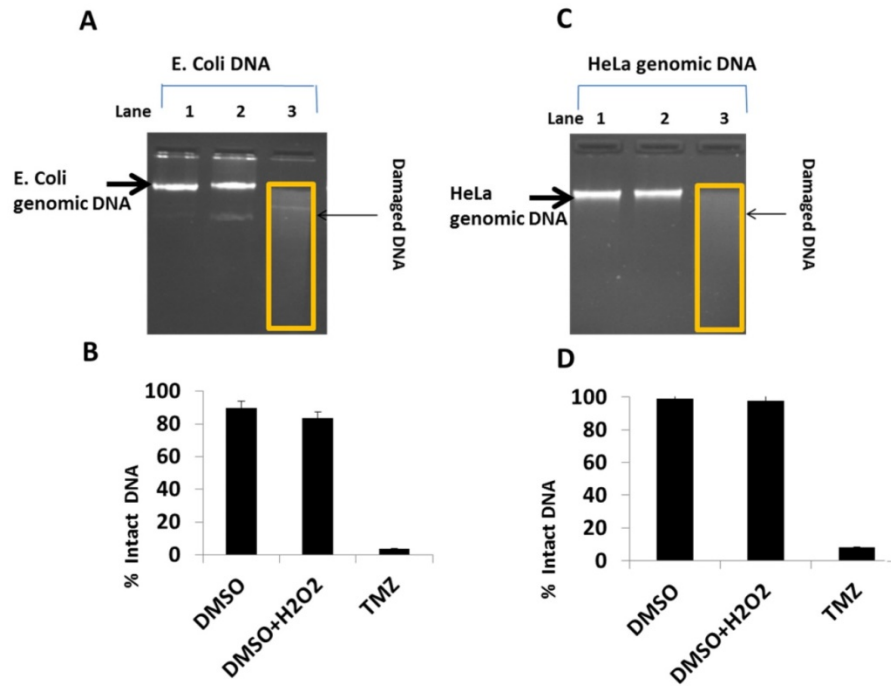


Figure 4. Action of TMZ on bacterial and animal cell genomic DNA. (4.A & 4.B) shows degradation of bacterial genomic DNA by TMZ in Lane 3 (Lane 1-Genomic DNA; Lane 2- H₂O₂ + DMSO; Lane 3-TMZ (1 mM)). Figure (4.C & 4.D) shows degradation of HeLa genomic DNA by TMZ; (Lane 1-Genomic DNA; Lane 2- H₂O₂ + DMSO; Lane 3-TMZ (1 mM)). Error bars represents mean ± SD.

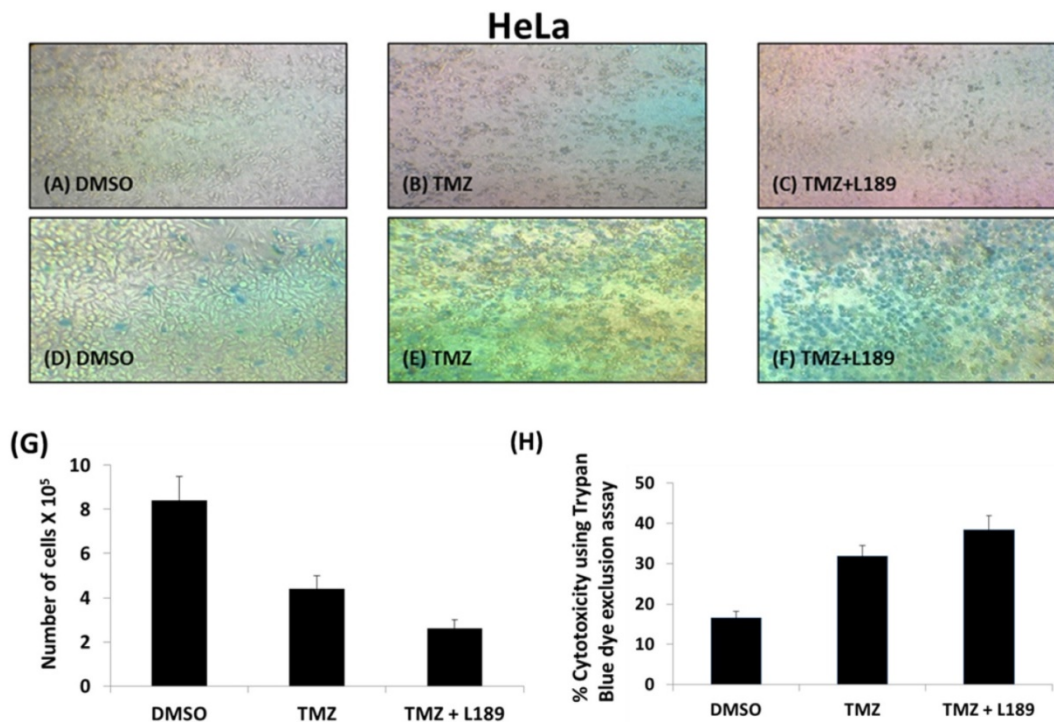


Figure 5. Cell based cytotoxicity of TMZ using trypan blue dye exclusion method. The microscopy photograph of unstained HeLa at 10X is provided in Figure 5 (A-C). The Figure 5 (D-F) shows the Trypan Blue dye stained HeLa directly in the culture dish. Each experiment was conducted independently three times. The total cell count and cytotoxicity data obtained using Trypan blue dye exclusion assay. The Figure 5G depicts the total cell count using the hemacytometer. The percentage of HeLa cytotoxicity due to TMZ and L189 treatment is given Figure 5H. Error bars represents mean ± SD.

Cell cytotoxicity of TMZ using trypan blue dye exclusion assay

Based on appreciable DNA single-strand break activity of TMZ, authors performed *in vitro* cell cytotoxicity assay to evaluate their abilities against HeLa. The Trypan blue dye exclusion data show the decrease in the cell count in TMZ treated cells. Further, cell death count is reduced drastically due to combined treatment of TMZ & L189 in HeLa compared to DMSO control. The microscopy data of treated or untreated HeLa are presented in Figure 5 (A-C). The photomicrography after Trypan blue stained cells also confirm the increase in cell death by the uptake of the dye as given in Figure 5 (D-F). The analysis of microscopy data suggest that TMZ and TMZ in combination with L189 treatment bring some extent of cytotoxicity, but not appreciable. The quantitative analysis of total cell count, viability and percentage cytotoxicity is presented in Figure 5G and 5H. The data indicate that total cell count in HeLa significantly reduced by the addition of L189 compared to TMZ alone. However, author notice that extent of cell cytotoxicity is not altered comparing TMZ alone or TMZ with L189. The data obtained from trypan blue dye exclusion assay points out that L189 make noticeable contribution to bring down the HeLa cell proliferation.

Determination of apoptotic DNA fragmentation

In literature, several anti-cancer drugs are reported to show cell cytotoxicity based due to ability to elicit apoptosis and presence of nuclear DNA fragmentation. Here, authors conducted apoptotic DNA fragmentation assay using well accepted protocol as mentioned in the method section. The ethidium bromide stained gel of apoptotic DNA samples extracted from HeLa with or without drug/inhibitor treatment is presented in Figure 6. The observations of data clearly indicate the absence of no apoptotic DNA fragments in HeLa genomic DNA. However, DNA quantification of genomic DNA explains and support cell based cytotoxicity data. The absence of apoptotic DNA fragments may be explained due to the presence of robust repair mechanism against TMZ mediated single-strand DNA break in HeLa. The analysis of gel indicates that L189 reduced the total amount of genomic DNA content in HeLa and supported by the cell count data using microscopy and cell count assay.

Discussion

Many anticancer drugs and radiotherapy act by modifying a drug target and inducing cell cytotoxicity

further leading to metabolic or apoptosis based cell death [3-6]. A number of potential anticancer drugs are classified as DNA genotoxic agents. Chemotherapeutic agents like alkylating agent TMZ, which are known to cause DNA damage like single or double strand break by modifying DNA sequences non-specifically can be coupled with participating DNA repair protein inhibitors to overcome such less responsiveness [5, 10].

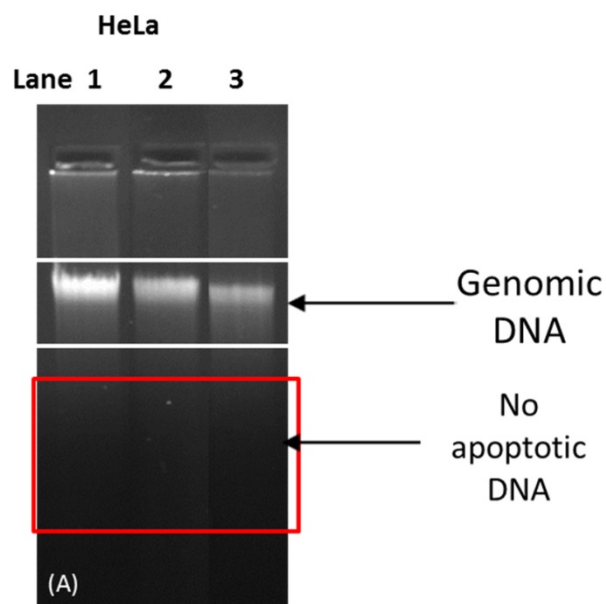


Figure 6. Determination of fragmented DNA by Apoptotic DNA Isolation. (Lane 1- control, lane 2- TMZ, lane 3- TMZ + L189). The figure depicts that genomic DNA content of HeLa with no apoptotic DNA.

DNA damages are caused by many chemotherapeutic agents and carcinoma escapes the cell death using their DNA repair machinery [30]. TMZ, an anticancer drug is reported by alkylating the DNA in certain cancer types including glioblastoma multiforme and prostate cancer. The DNA alkylation pattern mainly encompass the N⁷ and O⁶ positions of guanine, and the O³ position of adenine, methylation of O⁶ guanine residue (O⁶-MG) [22, 31-33]. Till date, evidences of TMZ as a single-strand DNA damaging agent are lacking on various DNA substrates. The study is carried out to check DNA nicking on various DNA substrates like plasmids, bacterial and HeLa DNA with TMZ at various conditions that is at different concentrations and temperature. Earlier reports indicate that the concentration range of drug TMZ varied from 0.3 mM to 2.5 mM [25]. Results interpret the three forms of DNA as supercoiled DNA as the lower band, the nicked or relaxed DNA as the uppermost band and linearized DNA in the middle. The maximum nicking of the plasmid DNA is observed at 1 mM and was considered optimum for

further experiments. The present results are supported by the earlier finding showing other alkylating agents as DNA damaging agents. Their observations point out that alkylation modification of DNA bases on double stranded plasmid DNA may be responsible for the destabilization of double stranded DNA secondary structure [34, 35]. As point of concern, MMR system is not available during *in vitro* DNA damage assay over cell cytotoxicity assay. However, authors defend current observations that state of double stranded DNA during cell cytotoxicity assay are different from *in vitro* DNA damage assay. Inside cell, double stranded DNA is not naked surrounded by a number of DNA binding proteins. Therefore, behavior of DNA is displayed differently compared to *in vitro* DNA damage assay with exposed plasmid DNA and destabilized secondary structure. Findings suggest that destabilization of plasmid DNA structure linked with created nicked/cleaved strand even in the absence MMR system.

The use of plasmid DNA substrate is not reported in the literature with reference to TMZ single-strand DNA break activity in different time point. Hence, experiments are carried out at various time points using TMZ as DNA damaging drug and plasmid DNA substrate. The similar experimental methods are reported to use plasmid DNA substrate to establish nicking activity of anticancer drugs [25]. Literature suggests that prolonged exposure of TMZ is able to induce cellular stress and cytotoxicity [36]. Experiments were carried out at 37°C with different time points and the results reveal that maximum relaxation of plasmid DNA occurred at 1 mM TMZ concentration for 24 h reaction time. The present experimental time point up to 24 h is comparable with other reported research documenting 24 and 48 h.

The TMZ is dissolved in organic solvent DMSO for the prolonged storage and stability. DMSO, an anti-oxidant agent is reported to scavenge free radicals that may harm or alter the DNA structure. The data indicate that DMSO could not interfere with the DNA damaging effects of TMZ. At the same time H₂O₂ is reported as agent to cause DNA damage by generating free radicals and facilitating mutagenesis and single and double strand break [37]. In our settings, the damaging activity of H₂O₂ is quenched by DMSO solvent. Conversely, plasmid DNA treated with TMZ show dramatically enhanced DNA damage. The TMZ lacks sequence specificity and alkylate on number of sites causing damage to the DNA. At present, evidences are available to validate that TMZ hinge on DNA methylation specifically at the N-3 of adenine and N-7 position of guanine or O-6 positions of guanine residues. Further, earlier findings support that methylation of DNA cell culture model,

single-strand break observed due to TMZ effects. As, TMZ brings damage to both plasmid and genomic DNA pointing out that TMZ *in vitro* DNA damage activity may not be substrate specific.

In literature, findings suggest about several mechanisms for TMZ mediated cytotoxicity and cell death. To evaluate and quantify the cell viability, Trypan blue dye exclusion and MTT assay are routinely carried out. Trypan blue dye exclusion assay is a basic and simple cell based assay to study cell viability. The use of this assay is also appreciated to determine the total cell content and in turn to assess the cell proliferation rate [38, 39]. Studies report the cytotoxic and anti-proliferative activity of triazene compounds similar structure with TMZ in several carcinomas including HeLa [40, 41]. At present, the TMZ concentration in cell cytotoxicity assay is supported from earlier reports of Liu *et al.* (1999) [18] that TMZ in the range of 100 µM to 2 mM creates single-strand break in the appropriate cell lines. Further, they delineate the option to convert single-strand DNA break into lethal double strand break DNA repair protein inhibitors. Furthermore, Yamauchi *et al.* (2008) [13] show that use of TMZ in cell cytotoxicity is used up to 1 mM. In our findings, HeLa were significantly responsive when treated with combination of TMZ & L189.

TMZ a potent alkylating agent is responsible for induction of autophagy that constitutes degradation of genetic material via formation of autophagosome rather than opting for apoptosis in glioma cells [42-44]. During apoptotic DNA fragmentation assay, result does not mark the presence of apoptotic DNA. Instead of, the genomic DNA is reduced in HeLa after treatment with TMZ and inhibitor. Present finding suggests that such observations can be due to the DNA repair protein complex of HeLa repairing the damage caused by the drug or by induction of autophagy that bypassed the apoptotic phase. Several anti-cancer drugs are also reported to work along with small molecule inhibitors DNA repair proteins [5, 10, 30]. Earlier findings clearly substantiate that DNA ligases including ligase III protein levels are reported to be increased in several types of carcinoma including HeLa [45, 46]. Hence, findings suggest that in HeLa, DNA ligase inhibitor L189 treatment may improve cytotoxicity of TMZ by interfering with DNA ligase action and stalling BER. Further, efforts are pursued to explore molecular mechanisms behind differential responses of combined action of L189 and TMZ in HeLa.

Conclusions

In conclusion, findings report TMZ as a single-strand breaking agent against different DNA

substrates. Further, data suggest that TMZ alone is less effective in HeLa. But, L189 added with TMZ produced significant anti-proliferative response in HeLa with mild toxicity. Hence, data point out that TMZ may produce indirect single-strand break and cytotoxic effects in HeLa due to blocked action of DNA ligases by L189.

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Authors' Contributions

Jahagirdar D: Contributed to data collection and compilation. Purohit S: Contributed to collection and compilation of information. Sharma NK: Conceived idea, title, draft and final writing of manuscript.

Competing Interests

The authors have declared that no competing interest exists.

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