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Original Article

Cyto-genotoxicity Assessment of Potential Anti-tubercular Drug Candidate Molecule-trans-cyclohexane-1, 4-diamine Derivative-9u in Human Lung Epithelial Cells A549

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ABSTRACT

Increasing incidences of multiple drug-resistance (MDR) in *Mycobacterium tuberculosis* are emerging as one among the serious public health threats and socio-economic burden to the third world countries including India. Last couples of decades are witnesses of the dedicated and sustained efforts made toward the development of target specific and cost-effective antimicrobial agents against MDR-*M. tuberculosis*. However, the drugs in use are still incapable of controlling the upsurge of MDR. Thus, in order to address the issue, we synthesized a library of symmetrical trans-cyclohexane-1, 4-diamine derivatives and evaluated their anti-mycobacterium activity in H_{37}RV strain of *M. tuberculosis*. A range of efficacy has been recorded in different derivatives of synthesized compounds and compound "9u" having i-propyl group substitution at p-position, was found to have more significant detrimental effects against the tested strain of *M. tuberculosis*. The present investigations were aimed to study whether the effective anti-mycobacterium concentrations of "9u" are biologically safe to human cells or not? The human lung epithelial cell line-A549 were exposed to a range of concentrations, i.e., at and above the anti-mycobacterium effective dose of "9u" for a period of 0-96 h. The standard endpoints of cytotoxicity viz., tetrazolium bromide salt (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide), neutral red uptake, lactate dehydrogenase release, trypan blue dye exclusion assays; and genotoxicity viz., micronucleus and chromosomal aberrations assays were used to evaluate the bio-safety of test compound. The compound "9u" shows no significant cytotoxicity and genotoxicity in A549 cells exposed to 10^{-5} M for 72 h, a concentration substantially higher than the concentration kill the H_{37}Rv strain of *M. tuberculosis.* The compound 9u was found to be safe up to 10^{-4} M if given for 24 h. The data reveal the therapeutic potential of compound 9u against *M. tuberculosis* without any having any cytotoxicity and genotoxicity responses.

Key words: A549 cell line, chromosomal aberration, cytotoxicity, micronucleus assay, *Mycobacterium tuberculosis*

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INTRODUCTION

Tuberculosis is one among the leading cause of morbidity and mortality in third world countries, including India and has imposed a significant socio-economic burden to society. The number of patients and financial burden are increasing exponentially every year due to the upsurge in the multiple drug-resistance (MDR) in the causal

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organism i.e., *Mycobacterium* spp.[1,2] Plasmids are known to harbor the drug-resistance genes in most of the pathogenic bacteria as their primary line of defense and *Mycobacterium tuberculosis* is not an exception. The MDR in *M. tuberculosis* against the available therapeutic agents is reported largely due to high mobility of such R-plasmids.[3] This infectious disease is usually found to be associated with a number of confounding factors such as socio-economics, hygiene, malnutrition and down the line impaired immune status, etc., The other prominent reason for being long persistence of this facultative intracellular pathogen in the body that it's multiplication in the host phagocytic macrophages, leading to poor availability of drugs.[4-7] Besides this, the long treatment period (6-12 months) of therapeutic agents viz., rifampicin, isoniazid (ISN), ethambutol and pyrazinamide, etc., has also been reported to induce moderate to severe hepatotoxicity, renal toxicity.[8] To overcome the issue of toxicity, effective and localized drug delivery systems were used and found to reduce the treatment regimens and/or dosage, as well as increased the bioavailability of the drugs. Hence, people have suggested the local drug delivery to the intracellular lung epithelial cells and macrophages in which the tubercle bacilli, as the ideal way of treating the tuberculosis without toxicity and/or low side-effect.[9,10] Though, such highly effective regimens have been developed to treat the tuberculosis patients, but still the duration of drugs is for a minimum of 6 months to cure the disease completely. The non-adherence to this lengthy treatment is reported as one among the major causes of MDR and extensively drug-resistance to *Mycobacterium* strains, which eventually complicates the treatment schedule and cure of the disease.[11,12] Reports are there showing the development of drug-resistance in tuberculosis patients due to altered pharmacokinetics because of other systemic disorders including acquired immunodeficiency syndrome.[13,14] Therefore, the need of the hour is to develop new anti-tuberculosis drugs, which could be targeted to specific cellular pathways, combating to drug-resistance, shorten and/or simplify current treatment regimens, provide effective therapy for patients intolerant to current first-line drugs and also provides the treatment to patients with latent *Mycobacterium* infection. In the recent past, a series of the symmetrical and asymmetrical cyclohexane-1,2-diamine[15,16] and cyclohexane-1,3-diamine derivatives[17] have been synthesized and reported to have significant antibacterial activity against both Gram-positive and Gram-negative bacteria with very low amount of toxicity. The anti-mycobacterial activity of the best active symmetrical and asymmetrical derivatives against *M. tuberculosis* $H_{37}Rv$ strain ranges between 3.125 and 12.5 mM.[18]

We have also synthesized a library of symmetrical trans-cyclohexane-1,4-diamine and evaluated them against *M. tuberculosis* $H_{37}Rv$ stain to assess their applicability as potential anti-tuberculosis drug candidate molecules. Out of the 27 compounds tested, four compounds were having significant activity against *M. tuberculosis* $H_{37}Rv$ stain. Compound "9u" having i-propyl group substitution at p-position was found to be the most potent among all the tested compounds.[19] In order to evaluate the therapeutic applicability of compound "9u", showing highly significant activity against *M. tuberculosis* $H_{37}Rv$ stain, toxicity evaluation in the target host cells is also a primary concern. So, the present studies were carried out to investigate the biological safety of compound "9u" at and above the anti-tuberculosis effective dose in the target organ cells. The biological safety was evaluated using standard endpoints of cytotoxicity and genotoxicity in human lung epithelium cells-A549.

MATERIALS AND METHODS

Reagents and consumables

All the specified chemicals and reagents were purchased from Sigma (Sigma St. Louis, MO, USA) unless otherwise stated. Culture medium Dulbecco's Modified Eagle Medium/ Nutrient Mixture F-12 (DMEM/F-12), antibiotics, fetal bovine serum (FBS) and trypsin-Ethylenediaminetetraacetic acid were purchased from Gibco BRL, USA. Culture wares and other plastic wares used in the study were procured commercially from Nunc, Denmark. Milli Q water (double distilled, deionized water) was used in all the experiments.

Cell culture

Human lung A549 cell line used in the study was procured from National Centre for Cell Sciences, Pune, India and maintained at *in vitro* Toxicology Laboratory, CSIR-Indian Institute of Toxicology Research, Lucknow, India, as per the standard protocols. In brief, the cells were cultured in DMEM/F-12, supplemented with 10% FBS, 0.2% sodium bicarbonate, 100 units/ml penicillin G sodium, 100 μg/ml streptomycin sulfate and 0.25 μg/ml amphotericin B. Cultures were maintained at 37°C at 5% CO_2-95% atmosphere under high humid conditions. Medium was changed twice weekly and cultures were passaged at a ratio of 1:6 once in a week. Prior to use in the experiments, cell viability was ascertained by trypan blue dye exclusion assay. The culture, showing viability more than 95% were used in all the experiments. All the experiments were done on the cells with passage 18-25 only.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay

Non-cytotoxic doses of compound "9u" were identified in A549 cell line (human lung epithelial cell line). Cytotoxicity assessment was done using standard endpoint i.e., tetrazolium bromide MTT assay following the protocol of Kashyap *et al*. 2010.[20] In brief, A549 cells (1×10^4 cells/well) were seeded in 96-well tissue culture plates and incubated in the CO_2 incubator for 24 h at 37°C. Then the medium was

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aspirated and cells were exposed to medium containing Compound (10^{-8}-10^{-3} M) for 24-96 h at 37°C in 5% CO_2-95% atmosphere under high humid conditions. Tetrazolium salt (10 µl/well; 5 mg/ml of stock in phosphate-buffered saline [PBS]) was added 4 h prior to completion of respective incubation periods. At the completion of the incubation period, the reaction mixture was carefully taken out and 200 µl of culture grade dimethyl sulfoxide was added to each well. The content was mixed well by pipetting up and down several times until dissolved completely. Plates were then incubated for 10 min at room temperature and color was read at 550 nm using multi-well microplate reader (Synergy HT, Bio-Tek, USA). The unexposed sets and sets exposed to $MnCl_2$ (10^{-3} M) were also run parallel under identical conditions that served as a basal and positive control respectively.

Neutral red uptake assay

The assay was carried out following the protocol described earlier by us.[21] Cells were exposed to compound in identical experimental setup as to MTT assay. Upon the completion of the incubation period, the medium was aspirated and NRU salt (50 µM/ml in the medium) was added 100 µl per well plate and incubated for 3 h. Then, the reaction mixture was carefully taken out and plates were washed with washing solution (100 µl/well) containing 1% $CaCl_2$ (w/v) and 0.5% HCHO (v/v) for remove unincorporated dye. Washing solution was removing and a mixture of 200 µl 1% acetic acid and 50% ethanol was added. The plates were kept on rocker shaker for 10 min at room temperature and then analyzed at 540 nm using multi-well micro plate reader (Synergy HT, Bio-Tek, USA). Unexposed sets were also run under identical condition and served as controls.

Lactate dehydrogenase release assay

LDH release assay is a method to measure the membrane integrity as a function of the amount of cytoplasmic LDH released into the medium. The assay was carried out using ready-made commercially available LDH assay kit for *in vitro* cytotoxicity evaluation (TOX-7, Sigma St. Louis, MO, USA). The assay was based on the reduction of NAD by the action of LDH. The resulting reduced NAD (NADH+) was utilized in the stoichiometric conversion of a tetrazolium dye. The resulting colored compound was measured using the multi-well plate reader at wavelengths 490 and 690 nm. In brief, the cells were exposed to (10^{-8}-10^{-3} M) for different time periods after the completion of the respective time periods the cells were processed for LDH release assay similar to MTT assay. Culture plates were removed from CO_2 incubator as per the experimental schedule and centrifuged at 250 × g for 4 min. Then supernatant of each well was transferred to a fresh flat bottom 96 well culture plate and processed further for enzymatic analysis as per the manufacturer's instructions.

Cell viability by trypan blue dye exclusion assay

The test was conducted to study the cell viability by assessing the loss of membrane integrity following the method of Pant *et al*. 2001.[22] In brief, the cells (4×10^4/well) were seeded in 48 well culture plates and allowed to grow for 24 h in 5% CO_2-95% atmosphere at 37°C under high humid conditions. Then the medium was replaced and exposed to compound (10^{-8}-10^{-3} M). Following the compound exposure, cells were subjected to assess the loss of cell viability. Immediately after the completion of the respective time periods, cells suspensions were aspirated and centrifuged at 600 rpm for 5 min and washed twice with sterile PBS (pH: 7.4) and re-suspended in the small amount of PBS. The cell suspension was then mixed with trypan blue dye (0.4% solution) at a ratio of 1:5 (dye: Cell suspension) and placed in hemocytometer. The counting to live (unstained, transparent) and dead (blue stained) cells were made at cell counter (Invetrogen, USA). The parallel sets were also run under identical conditions and served as controls.

Micronucleus assay

MN assay was carried out using standard protocols Srivastava *et al*. 2010.[23] Briefly, A540 cells were grown on cover slips placed in 8-well plates in DMEM/F-12 medium. The cells were exposed to different concentration of the compound; cells were incubated up to 43-44 h in fresh medium and blocked for cytokinesis using cytochalasin-B (3 µg/ml). Cells were then harvested by hypotonic buffer (0.075 M KCl) for 5-10 min at 37°C and fixed in Carnoy's fixative (methanol/acetic acid, 3:1). Finally, cells were dropped onto the slides and stained with 5% Giemsa in phosphate buffer (pH: 6.8) for 15-20 min and mounted with DPX for microscopic examination. A minimum of 1000 binucleated cells with well-defined cytoplasm in each slide was scored for the presence of MN using a Nikon Eclipse 80i upright microscope attached to a Nikon digital CCD cool camera (Model DS-Ri1 of 12.7 Megapixel). Data presented for MN are the mean of three slides.

Chromosomal aberration assay

A549 cells were cultured in DMEM/F-12 medium in 25 cm^2 flasks and the cells were exposed to different concentration of the compound. Colcemid (0.15 µg/ml final concentration) was added 4 h prior to harvest the cells. After the compilation of 4 h exposure of colcemid, the medium was aspirated and the cells were washed with Hank's balanced salt solution. After that the cells were given a hypotonic shock in potassium citrate (0.8%) for 30 min. Finally, cells were fixed in cold fixative (methanol: acetic acid, 5:2 ratios). Cells were dropped on clean slides, dried and stained in 5% Gurr's Giemsa. The cells were scored for the presence of CA using a Nikon Eclipse 80i upright microscope attached

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to a Nikon digital CCD cool camera (Model DS-Ri1 of 12.7 Megapixel). Data presented for CA are the mean of three slides.

RESULTS

MTT assay

Results findings of MTT assay are summarized in Figure 1. Human lung epithelium cells-A549 responded to test compound in a concentration and time dependent manner. There was no significant reduction in percent cell viability reported all through the exposure period, i.e., till 96 h in any of the used concentration i.e., 10^{-8}-10^{-5} M of the test compound 9u. Whereas, higher concentrations of compound used, i.e., 10^{-3} M were found to cause a gradual reduction in percent cell viability, which reaches to significant levels at and above the exposure period of 48 h. The cell viability reduces to 93 ± 1.15, 85 ± 2.31, 78 ± 1.15 and 70 ± 2.31 in the cells exposed to 10^{-4} M for a period of 24, 48, 72 and 96 h respectively. The reduction was severe in cells exposed to the highest concentration, i.e., 10^{-3} M, where the viability reduces to 77 ± 1.73, 61 ± 1.15, 47 ± 2.31-33 ± 1.15 at 24, 48, 72 and 96 h respectively, when compared with unexposed control cells [Figure 1].

NRU assay

The observations of NRU assay were having similar trends as that to the MTT assay [Figure 2]. The lower concentrations, i.e., 10^{-8}-10^{-5} M were found to induce no significant reduction in percent cell viability, while the higher concentrations, i.e., 10^{-5}-10^{-3} M were able to induce a dose dependent decrease in the percent cell viability, which reaches to statistically significant levels at and beyond 48 h and exposure, when compared with unexposed control cells [Figure 2].

LDH release assay

The highlights of the results for the release of LDH following the exposure of cells to compound "9u" are presented in Figure 3. A significant increase in LDH release was observed in 10^{-3} and 10^{-4} M in comparison to unexposed controls. The LDH release was increased significantly to 149 ± 4.04% at 24 h and it reaches its maximum up to 213 ± 4.62% at 96 h in cells exposed to 10^{-3} M concentration. The trends were similar after the exposure of 10^{-4} M and it shows 127 ± 2.31% at 24 h and reached up to 167 ± 2.31% at 96 h following exposure to test compound "9u." No significant changes were found in the cells exposed to 10^{-5}-10^{-8} M of test compound all through the exposure i.e., 96 h [Figure 3].

Trypan blue dye exclusion assay

The direct loss in the viable cell count was also assessed immediately after the exposure of compound at each time point (24, 48, 72 and 96 h) using trypan blue dye exclusion test. The result highlights are summarized in Figure 4. At the start of the experiment, under normal condition (unexposed control), the percent cell viability assumed as 100% of counted cells. The statistically significant reduction in percent viable cell count was started at 24 h. The gradual decrease in percent cell viability was continued in all further time periods (48, 72 and 96 h) i.e., 76.75, 63.5, 49.75 and 39.75% after the exposure of 10^{-3} M concentration of compound respectively and all these reductions were comparable to the values of unexposed control. In totality, the trend of cell viability loss was similar to that the other

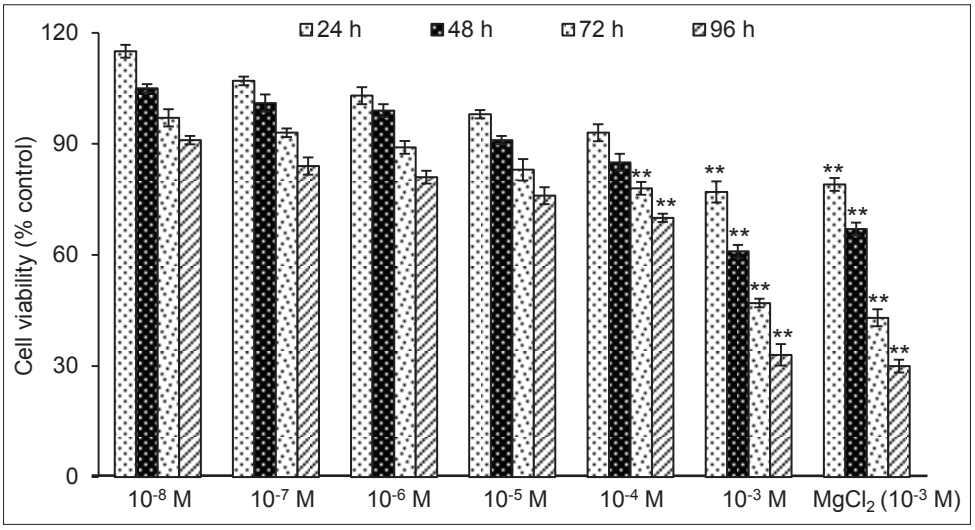


Figure 1: Cytotoxicity/biosafety assessment of potential anti-tubercular compound "9u" in human lung epithelium cells-A549 (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay. Cells were exposed to "9u" (10–3-10–8 M) for 24-96 h. the data presented are percent cell viability compared with unexposed control cells. Values are given as mean ± standard error of the data obtained from three independent experiments and each experiment contained at least eight replicates. *$P < 0.05$, **$P < 0.01$

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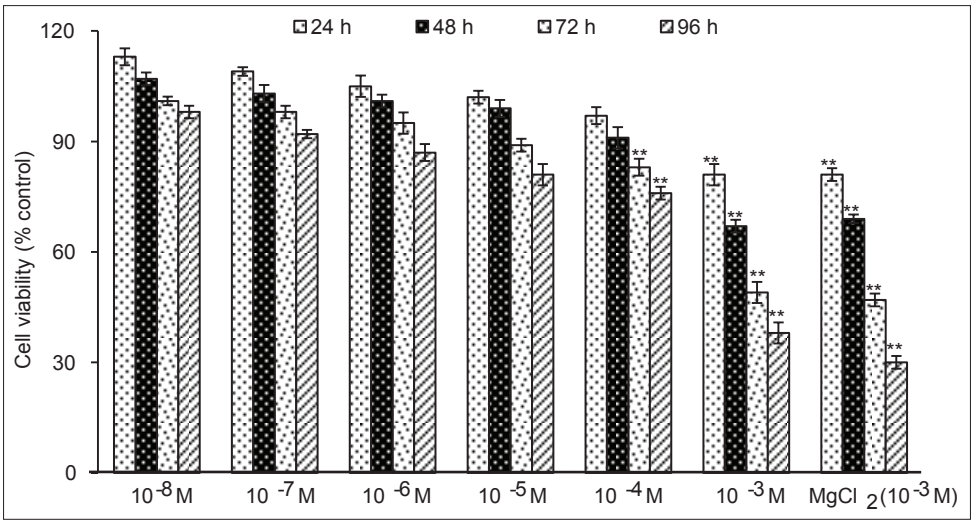


Figure 2: Cytotoxicity/biosafety assessment of potential anti-tubercular compound "9u" in human lung epithelium cells-A549 (neutral red uptake assay). Cells were exposed to "9u" (10–3-10–8 M) for 24-96 h. the data presented are percent cell viability compared with unexposed control cells. Values are given as mean ± standard error of the data obtained from three independent experiments and each experiment contained at least eight replicates. **P* < 0.05, ***P* < 0.01

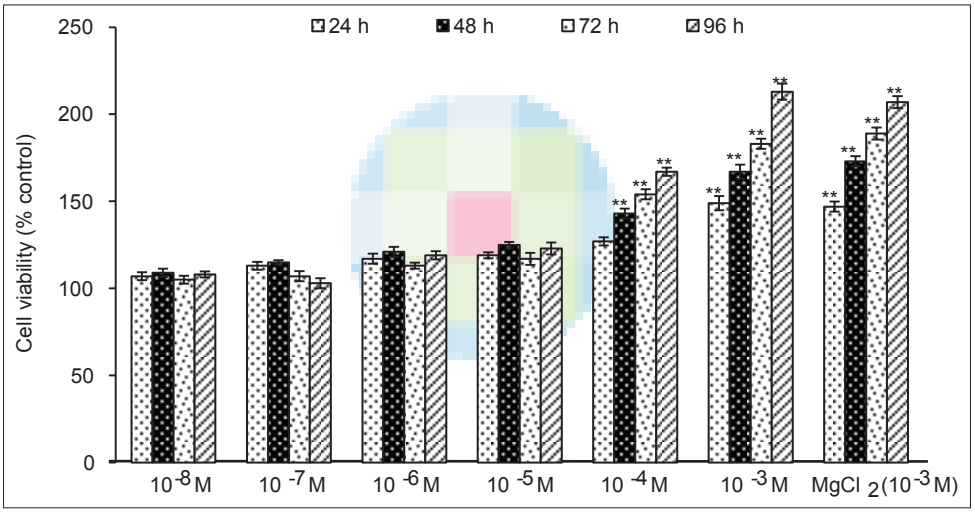


Figure 3: Cytotoxicity/biosafety assessment of potential anti-tubercular compound "9u" in human lung epithelium cells-A549 (lactate dehydrogenase release assay). Cells were exposed to "9u" (10–3-10–8 M) for 24-96 h. the data presented are percent cell viability compared with unexposed control cells. Values are given as mean ± standard error of the data obtained from three independent experiments and each experiment contained at least eight replicates. **P* < 0.05, ***P* < 0.01

tests carried out to assess the cytotoxicity i.e., MTT, NRU, LDH assays [Figure 4].

MN assay

MN assay was carried out to assess the accumulation of genetic damage in the cells. The cells were grown in DMEM/F-12 medium alone served as basal control and the other set of cells exposed to different concentrations of test compound "9u" for different time periods were considered as treatment groups. The increase in the MN frequency was observed as compared to the cells grow in normal medium [Figure 5]. We observed a significant increase in the induction of MN following the exposure of cells to 10^{-3} M of test compound, i.e., 15 ± 2.31, 21 ± 2.31, 26 ± 1.73 and 33 ± 1.15 MN/1000 cells at 24, 48, 72 and 96 h respectively. Whereas, the count of MN was insignificant in the cells exposed to 10^{-5}-10^{-8} M for all time period i.e., 24-96 h. The values of MN induction were also insignificant in the cells exposed to all the concentrations of test compound except 10^{-3} and 10^{-4} M [Figure 5].

CA-assay

The trends were similar as that to MN assay. The most common aberrations were found of chromatid gaps and break type, followed by higher concentrations of test compound. There were occasional incidences of

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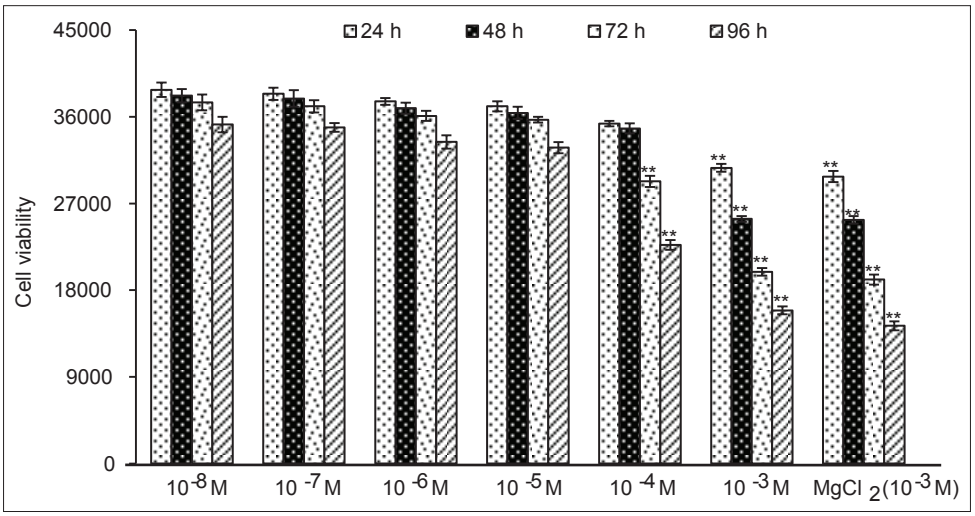


Figure 4: Cytotoxicity/biosafety assessment of potential anti-tubercular compound "9u" in human lung epithelium cells-A549 (trypan blue dye exclusion assay). Cells were exposed to "9u" (10–3-10–8 M) for 24-96 h. the data presented are percent cell viability compared with unexposed control cells. Values are given as mean ± standard error of the data obtained from three independent experiments and each experiment contained at least eight replicates. **P* < 0.05, ***P* < 0.01

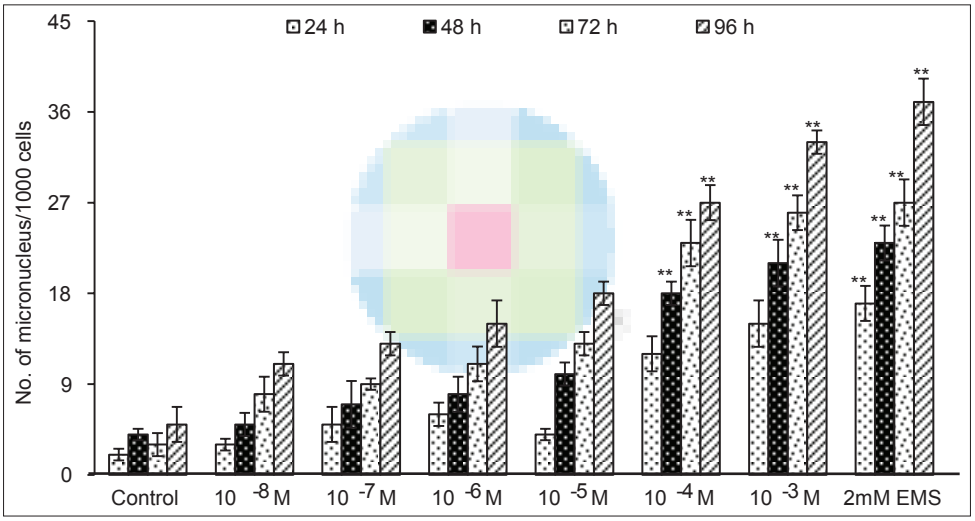


Figure 5: Genotoxicity/biosafety assessment of potential anti-tubercular compound "9u" in human lung epithelium cells-A549 (micronuclei assay). Cells were exposed to "9u" (10–3-10–8 M) for 24-96 h. Micronuclei were calculated by scoring a minimum of 1000 cells at each time point, i.e. 24, 48, 72 and 96 h. Values are given as mean ± standard error of the data obtained from three independent experiments and each experiment contained at least eight replicates. **P* < 0.05, ***P* < 0.01

aneuploidy in the cells exposed to higher concentrations, i.e., 10^{-3} and 10^{-4} M and for longer duration i.e., 72 and 96 h. The induction of CA was dose dependent, i.e., 15 ± 1.73, 19 ± 1.15, 29 ± 2.31 and 37 ± 2.89 aberrations/100 in cells exposed to 10^{-3} M for 24, 48, 72 and 96 h respectively [Figure 6].

DISCUSSION

Bio-safety analysis of drug candidate molecules in the host cells using MTT, NRU, LDH, TBDE assays is a gold standard method and widely accepted as pre-screening in target specific cells host.[24,25] These cells based assays in drug discovery are well accepted as they are suggested as high-throughput screening tools to reduce the time.[26] The comparatively less common, but accepted methods also include the environmental assessment of chemicals[27] and biosensors for monitoring cellular behavior.[28]

In the present investigations, we recorded the dose dependent toxic responses of test compound "9u" in human lung epithelium cells-A549 at very high doses, which are many fold higher than the effective doses against *M. tuberculosis* H_{37}Rv stain. The contrary to that Vahdati-Mashhadian *et al*. 2007[29] have reported a significant toxicity of rifampin in HepG2 cell line at an effective anti-tuberculosis dose 10 μM (8.23 μg/ml) and

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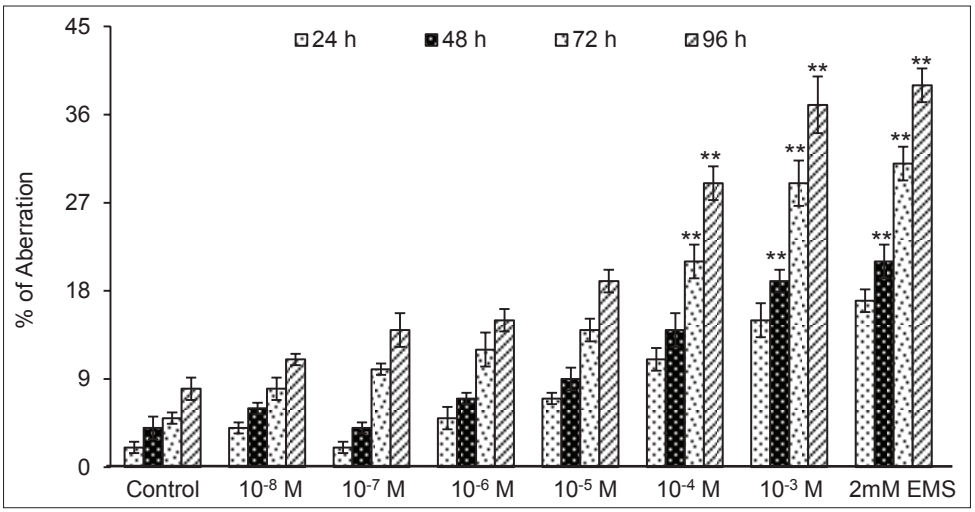


Figure 6: Genotoxicity/biosafety assessment of potential anti-tubercular compound "9u" in human lung epithelium cells-A549 (chromosomal aberration [CA]). Cells were exposed to "9u" (10–3-10–8 M) for 24-96 h. CA were scored at 24, 48, 72 and 96 h in the cells exposed to test compound "9u". Values are given as mean ± standard error of the data obtained from three independent experiments and each experiment contained at least eight replicates. $^{*}P < 0.05$, $^{**}P < 0.01$

the magnitude of toxicity got increased with the increased concentration of rifampin. In a study carried out in similar fashion, Isefuku *et al*.[30] in their study have reported anti-proliferative activity of rifampin in osteoblast-like cells *in vitro* at concentrations equal or even lesser to that of effective concentration against *M. tuberculosis*. Though, in less magnitude, but rifampin induced hepato-toxicity is well reported in clinical settings too.[31] The reduced magnitude under *in vivo*/clinical conditions has been suggested due to the integrated system of the body where more than one organ is taking care of the toxicity/metabolism of xenobiotics.[32]

In our studies, less toxicity responses of test compound "9u" might be attributed because of high metabolic rate of the cells, which either allowed test compound a less time to stay or formed a less toxic/no toxic secondary metabolite(s) of the principle compound. While, HepG2 cells have the expression of a wide range of phase I enzymes such as cytochrome P450s (CYPs) and phase II enzymes and rifampin is known to metabolize into various active and inactive metabolites by the hepatic microsomes. Being a major metabolic site, liver cells, such as hepatocytes are well-documented to induce the CYP3A4, important cytochrome P450 enzyme responsible for the metabolism of foreign compounds.[32,33] These metabolized intermediates causes toxicity in the liver and kidney, which is the second extra hepatic site rich in metabolizing enzymes.

MN formation is an indication of fragmentation in chromosomes and that fragment is not incorporated into daughter nuclei during mitosis. Although, CA is referred as a missing, extra, or an irregular portion of chromosomal deoxyribonucleic acid, which is not integrated into daughter nuclei. The xenobiotics exposure had been demonstrated to form an atypical number of chromosomes or a structural abnormality in one or more chromosomes due to MN and/or CA. Our findings demonstrated that the test compound "9u" has no mutagenic potential even at many fold higher concentrations to that of effective against *M. tuberculosis* $H_{37}Rv$ stain. No significant induction in the MN and CA could be recorded, reported in any concentration of test compound except 10^{-3} and 10^{-4} M. While, rifampin was found to induce significant mutagenic responses in liver cells and erythrocytes in both blood and bone marrow of rats.[34] In a clinical investigation, Masjedi *et al*. 2000[35] observed a significant increase in the cytogenetic markers (MN and CAs) in pulmonary tuberculosis patients receiving treatment for 6 months, when compared to untreated and control groups. An increased frequency of MN and CA has also been reported in tuberculosis infection without any drug exposure.

Though, the experiments have not been carried out to study the specific binding of the test compound "9u" with the receptor or cytosolic receptor of the cells. That could be helpful to elaborate our hypothesis toward the exact mechanism(s) of cell-drug interaction and reasons of comparatively less toxicity than rifampin. We could have been hypothesized this since, *M. tuberculosis* is known to use as many as eight different cell surface receptors in host phagocytic cells and involved in survival, replication and pathogenesis of the bacteria.[36,37] Upon infection, mycobacteria reside within a specialized early phagosomal compartment. Pathogenic mycobacteria prevent fusion with the lysosome, which facilitates evasion of host bactericidal mechanisms and precludes the efficient antigen presentation.[38]

The major limitation with conventional therapy available today is long treatment period, i.e., minimum of 6 months. That daily chronic exposure of drugs for such a long period

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induces toxic responses in liver, kidney, lungs and other vascularized organs.[33] Apart from such toxic responses, the MDR in *Mycobacterium* spp. is another serious issue of concern.[39] In fact, natural products are supposed to have a small window of toxicity, with this view in mind and with supported literature, rifampin as a natural compound inhibiting the ribonucleic acid polymerase in *M. tuberculosis*, are being used as one of the safest and effective anti-tuberculosis agents. Though, it has comparatively less reported toxicity than ISN and pyrazinamide but certainly known for significant hepato-toxicity.[40-42] So, under these circumstances, our findings showing less/no toxic responses in the cells of target organs may be a step toward to develop the potential alternative therapeutic entities that can be used both to shorten the duration of therapy and to combat the growing problem of clinical drug-resistance.

CONCLUSION

Our result of *in vitro* studies carried out in human lung epithelium cells-A549 identifies the test compound "9u" biologically safe at the concentrations many fold higher than that of the effective concentrations against the activity of *M. tuberculosis* H_{37}Rv stain. The study also recommends the *in vivo* investigations using experimental models to further study the possible anti-tuberculosis potential of test compound "9u" at biological safe doses.

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