Original Article

Expression Profile of Markers of Apoptosis, Injury and Oxidative Stress in Human Lung Epithelium Cells-A5449 Receiving Chronic Exposure of Potential Anti-Tubercular Drug-*trans*-Cyclohexane-1, 4-Diamine Derivative-"9u"

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ABSTRACT

Earlier, we had reported the synthesis of a library of symmetrical trans-cyclohexane-1,4-diamine derivatives and evaluated them for their anti-mycobacterium activity in the H37RV strain of *Mycobacterium tuberculosis*. A range of efficacy was recorded in different derivatives and the compound "9u" having an i-propyl group substitution at the p-position was found to be the most effective. The compound "9u" was found to be safe for cytotoxic and genotoxic responses in human lung epithelium cells-A549, even up to many fold higher than that required to kill *M. tuberculosis.* Hence, compound "9u" was inferred to be a potential anti-tuberculosis drug of choice. However, the biological safety of compound "9u" upon chronic exposure was still to be answered because anti-tuberculosis (TB) treatment requires a minimum of 6 months' exposure of host systems and most of the available anti-TB drugs are known to induce apoptosis, oxidative stress and injury during such exposures. Thus, the present investigations were aimed to study the alterations, if any, in the expression profile (mRNA and protein) of markers associated with apoptosis, injury and oxidative stress in human lung epithelium cells-A549 receiving a chronic exposure of the potential anti-TB compound "9u." Our findings demonstrate that there was a statistically insignificant transient shift (until 3 weeks) in the markers of apoptosis, injury and oxidative stress, after which expression changes were similar to baseline, when compared with unexposed cells of respective time periods. The studied markers showed linearity in the trend at both mRNA and protein level, indicating the suitability of the test system selected in the study. The data confirm the therapeutic potential of compound "9u" for even long-term treatment against *M. tuberculosis* without having any significant apoptosis, injury and oxidative stress.

Key words: A549 cell line, apoptosis, injury, *Mycobacterium tuberculosis,* oxidative stress

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INTRODUCTION

Tuberculosis (TB) remains a major global health problem, particularly in many of the developing countries including India.[1] The disease is a slow progressive, chronic granulomatous infection caused by *Mycobacterium tuberculosis*, an intracellular pathogen that is capable of establishing and causing life-long infection in humans.

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About 9 million new cases of TB are reported all over the world, killing around 1.5 million people every year.[2] In India, approximately 12-15 million people are currently suffering from the disease, of which about half a million of the affected persons die each year. An increasing trend of the disease associated with the HIV infection has been recorded all over the world and, in India alone, of around 3.5 million HIV-infected persons, about 1.8 million individuals are co- infected with TB.[3]

The situation, however, has aggravated further with the emergence of multi-drug-resistant strains that have been detected to be resistant to all the major anti- TB drugs (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB) that was detected in 2006.[4] As the XDR-TB is resistant to several first- and second-line anti-TB drugs, the treatment options are limited and thus the mortality rates are extremely high. Therefore, the prevention of MDR-TB and XDR-TB must receive great emphasis, which might be achieved by the immediate institution of the treatment with more efficacious drugs. Recently, highly efficacious, inhalable drug delivery systems targeting macrophage-resident TB bacteria have been proposed.[5-9] Their efficacy has been ascribed to efficient drug targeting, which is not fully harmonized with the experience of high-dose chemotherapy for TB.[10,11] Inhaled biodegradable microparticles containing a large payload of anti-TB drugs[12] yield high intracellular drug levels[8] and evoke free radicals and tumor necrosis factor alfa secretion by murine macrophages.[7] Because microparticle phagocytosis might activate infected human macrophages,[13,14] whether apoptosis would be a result of such activation and whether it would be relevant to killing of the resident pathogen were examined. Subject areas have shown several phenomena relevant to the innate antimycobacterial activity and programmed cell death of infected macrophages[15,16] that were revisited to determine whether microparticle treatment would enhance the host defense strategy of macrophage apoptosis.

The induction of host cell death is an ancient immune defense strategy that is maintained throughout the animal and plant kingdoms. Virulent strains of *M. tuberculosis* employ several strategies to avoid the induction of macrophage cell death, and success in this process is clearly important for bacterial virulence. The molecular mechanisms of host cell apoptosis inhibition are little understood, but the recent identification of anti-apoptosis genes in the genome of *M. tuberculosis* has provided the tools necessary to investigate the details of this host-pathogen interaction. The results of these studies may prove useful for the development of new drug targets and/or vaccine candidates.[17]

Isoniazid (INH) is a first-line antibiotic used in the treatment of infections caused by *M. tuberculosis*. However, it has a serious limitation of being hepatotoxic. Delineating the mechanism underlying INH-induced hepatotoxicity may be beneficial in devising ways to counteract its toxic manifestations. Studies in human hepatoma HepG2 cells have indicated that INH exposure causes induction of apoptosis. HepG2 cells were exposed to increasing concentrations of INH (6.5, 13, 26 and 52 mM). Hydrogen peroxide (0.3 mM) served as the positive control. After incubating for specific time intervals, the cells were harvested and evidences of cytotoxicity, oxidative stress and apoptosis were sought. The findings indicated that INH exposure causes increased reactive oxygen species generation along with alteration in the levels of enzymatic antioxidants such as superoxide dismutase, catalase and glucose-6-phosphate dehydrogenase. Altered Bcl-2/Bax content, cytochrome-*c* translocation, caspase activation and DNA fragmentation emphasized involvement of apoptosis.[18]

In order to address the issue of cytotoxicity and apoptosis induced by existing anti-tuberculosis drugs, we have also synthesized a library of symmetrical trans-cyclohexane-1,4-diamine and evaluated them against the *M. tuberculosis* H37Rv stain to assess their applicability as potential anti-TB drug candidate molecules. Out of the 27 compounds tested, four compounds had significant activity against the *M. tuberculosis* H37Rv stain. Compound "9u" having an i-propyl group substitution at the p-position was found to be the most potent among all the tested compounds.[19] Further, the biological safety of compound "9u" to the host system was evaluated at and higher than the therapeutic doses in human lung epithelium cells-A549.[20] The present investigations were carried out to determine whether the anti-*M. tuberculosis* doses of compound "9u" impose any apoptotic/necrotic response in human lung epithelium cells-A549 following a long-term exposure or not.

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 DMEM/F-12, antibiotics, fetal bovine serum and Trypsin-EDTA were purchased from Gibco BRL, USA. All the antibodies used in this study were procured from Chemicon International, USA. Culturewares and other plasticwares 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 cell line A549 used in the study was procured from the National Centre for Cell Sciences, Pune, India and maintained at the *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% fetal bovine serum (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^{0}C in 5% CO_2 - 95% atmosphere under high humid conditions. The medium was changed twice weekly and cultures were passaged at a ratio of 1:6 once a week. Prior to use in the experiments, cell viability was ascertained by the Trypan blue dye exclusion assay. The cultures showing viability of >95% were used in all the experiments. All the experiments were performed on the cells with passage 18-25 only.

Drug exposure

Healthy growing A549 cells were exposed to the test drug i.e. *trans*-cyclohexane-1, 4-diamine derivative-"9u" at a therapeutic concentration (10^{-5}M) for a period of up to 7 weeks. Cells were harvested at an interval of 1 week to study the expression changes, if any, of markers associated with apoptosis, injury and oxidative stress. The cells were passaged as and when they reached the confluency. To keep the drug concentration constant, culture medium supplemented with drug was changed every alternate day.

Transcriptional changes

Drug-induced alterations in the mRNA expression of genes involved in the apoptosis, injury and oxidative stress were studied at an interval of 7 days up to a total of 7 weeks using the quantitative real time-polymerase chain reaction (PCR) technique. The drug-induced alteration in the levels of mRNA was expressed in relative quantification by comparing the data obtained from the unexposed cells. The quantitative real-time PCR analysis was performed by following the protocol described by us earlier.[19] In brief, total RNA was isolated from both experimental and unexposed control sets using GeneElute mammalian total RNA Miniprep Kit (Catalog No. RTN-70, Sigma, USA). Total RNA (1 µg) was reverse-transcribed into cDNA by SuperScript III first strand cDNA synthesis kit (Catalog No. 18080-051, Invitrogen Life Science, USA). Quantitative real-time PCR (RT-PCRq) assay reactions were carried out with 2X SYBR Green PCR master mix (Applied Biosystems, USA) using ABI PRISM 7900HT Sequence Detection System having software version 2.2.1 (Applied Biosystems, USA). Results were expressed relative to the housekeeping gene i.e. β-actin. Real-time reactions were carried out in triplicate wells for each sample.

Translational changes

The test compound "9u"-induced alterations in the expression of marker protein of apoptosis, injury and oxidative stress were also analyzed to establish the linearity in the changes at both transcriptional and translational levels. The alteration in protein expression was analyzed by Western blot analysis. The Western blot analysis was performed following the protocol described by us earlier.[20] In brief, cells were pelleted and lysed using Cell Lytic™ M Cell Lysis Reagent (Cat No# C2978, Sigma, USA) in the presence of protease inhibitor cocktail (Cat No# P8340, Sigma, USA). Protein estimation was performed using a BCA Protein Assay Kit (Cat No# G1002, Lamda Biotech, Inc., St. Louis, MO, USA). Then, denatured proteins (100 µg/well) were loaded and electrophoresed using 10% Tricine-SDS gel (Hermann Schagger, 2006). Proteins were transferred on polyvinylidene fluoride (PVDF) membrane (Millipore Cat No# IPVH00010, USA) by the wet transfer method at 180 mA current for 3 h. Non-specific binding was blocked with 5% non-fat dry milk powder in TBST [20 mM Tris-HCl (pH 7.4), 137 mM NaCl, and 0.1% Tween 20] for 2 h at 37°C. After blocking, the membranes were incubated overnight at 4^{0}C with anti-protein primary antibodies specific to the markers of apoptosis, injury and cell death (1:1000, Chemicon International, USA) in blocking buffer (pH 7.5). The membranes were then incubated for 2 h at room temperature with secondary anti-primary antibody conjugated with horseradish peroxidase (Chemicon International, USA). Then, the blots were developed using the Super Signal West Fempto Chemiluminescent Substrate™ (Thermo Fisher Scientific, USA) and Bio-Rad Versa Doc™ Imaging System 4000 (Bio-Rad, PA, USA). The densitometry for protein-specific bands was performed in a gel documentation system (Alpha Innotech, USA) with the help of AlphaEase™ FC Stand Alone V.4.0 software. Actin-β was used as the internal control to normalize the data. "9u"-induced alterations are expressed in relative term fold change in expression by comparing the data with respective unexposed controls.

Statistical analysis

The results are expressed as mean and standard error of means (Mean ± SE) for at least three experiments. One-way ANOVA followed by *post hoc* Dunnett's test was employed to detect differences between the groups of treated and controls. $P < 0.05$ was taken to indicate significant differences.

RESULTS

Transcriptional changes (^{q}RT-PCR studies)

Result highlights of test compound "9u"-induced alterations in the mRNA expression of markers associated with apoptosis, injury and oxidative stress are presented in Figure 1a - c. There was a continuous increase in the mRNA expression in pro-apoptotic genes, i.e. bax (1.4 ± 0.12, 1.5 ± 0.06 and 2.1 ± 0.10), p53 (1.2 ± 0.08, 1.4 ± 0.07

and 1.8 ± 0.05) and caspase-3 (1.3 ± 0.06, 1.6 ± 0.09 and 1.9 ± 0.08) and downregulation in anti-apoptotic gene bcl2 (0.93 ± 0.06, 0.90 ± 0.05 and 0.85 ± 0.03) in the first 3 weeks in the cells exposed to test compound 10^{-5} M of "9u" when compared with the unexposed control cells of respective time periods. Although the drug-induced alterations were observed in the first 3 weeks, these alterations did not reach significance. From the fourth to seventh weeks, altered values were found to be brought back toward the basal level for all the markers of apoptosis [Figure 1a]. Similar to that of apoptosis markers, genes associated with cell injury were also found to follow similar trends. The levels of mRNA of b-FGF (1.2 ± 0.01, 1.5 ± 0.04 and 2.1 ± 0.07), JNK (1.4 ± 0.03, 1.7 ± 0.03 and 2.3 ± 0.08), C-Jun (1.7 ± 0.06, 2.1 ± 0.08 and 2.6 ± 0.09) and C-Fos (1.5 ± 0.05, 1.8 ± 0.05 and 2.4 ± 0.06) in first 3 weeks were found to be increased in exposed cells in comparison with unexposed cells and thereafter the levels were down to the basal levels by the seventh week of incubation [Figure 1b]. Following a similar trend, markers of oxidative stress were altered in the initial weeks but came back to the basal level in cells incubated with drug for a longer period of time [Figure 1c]. The results indicated that the drug "9u" induced a transit non-significant alteration (up to 3 weeks) in the markers of apoptosis, injury and oxidative stress in human lung epithelium cells-A549, which could be recovered back upon the chronic exposure of the test compound, i.e. 7 weeks.

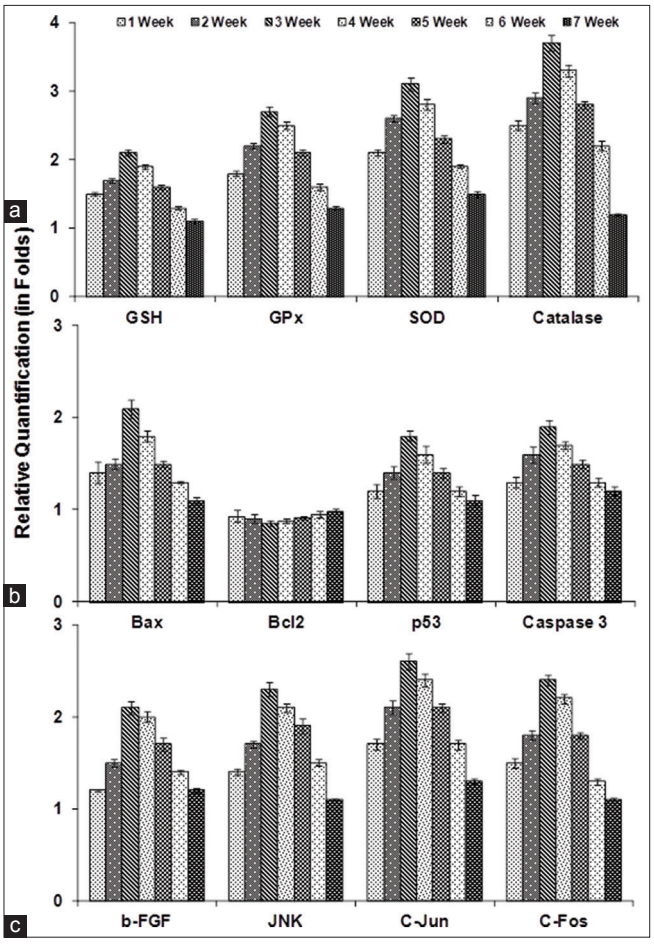

Figure 1: Transcriptional changes (quantitative real-time polymerase chain reaction analysis) of genes associated with apoptosis, injury and oxidative stress in human lung epithelium cells-A549 exposed to potential anti-tubercular compound "9u." (a) Alteration in mRNA expression of marker genes of apoptosis, (b) alteration in mRNA expression of marker genes of injury and (c) alteration in mRNA expression of marker genes of oxidative stress. Values are given as mean ± standard error of the data obtained from three independent experiments and each experiment containing at least three replicates. Actin-β was used as the internal control to normalize the data and "9u"-induced alterations in mRNA expression are expressed in relative quantity compared with the respective unexposed control groups. Reliability of specific products was checked by melting curve analysis as well as running the product onto 2% agarose gel

Translational changes (Western Blot Analysis)

The data of the protein expression changes carried out by Western blot analysis have shown linearity with the transcriptional changes. The representative data of Western blot analysis of protein expression of markers of apoptosis, injury and oxidative stress are presented in Figure 2a - c. The test compound "9u" was found to influence the upregulation of pro-apoptotic genes bax (1.20, 1.48 fold of control), caspase-3 (1.24. 2.06) and p53 (1.82, 2.55 fold of control) and downregulation of anti-apoptotic gene bcl2 (0.86, 0.70 of control) in the first and third weeks, while by the seventh week most of the markers were close to the unexposed control levels. The alterations observed in the first and third weeks were also non-significant statistically [Figure 2a]. The results for drug-induced

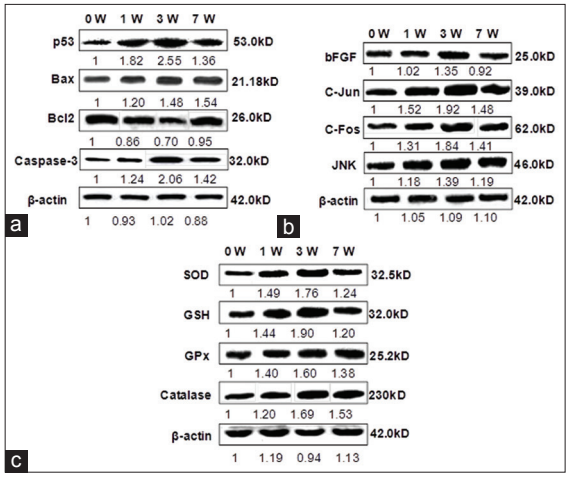

Figure 2: Translational changes (Western blot analysis) of protein markers associated with apoptosis, injury and oxidative stress in human lung epithelium cells-A549 exposed to potential anti-tubercular compound "9u." (a) Alteration in the protein expression of markers of apoptosis, (b) Alteration in the protein expression of markers of injury and (c): Alteration in the protein expression of markers of oxidative stress. The data are representative of three independent experiments and each experiment contained at least three replicates

alterations in protein expression of markers of injury had similar trends as that of apoptotic markers. However, the magnitude of alteration was slightly lower than that of the apoptotic markers. But, in both cases, the changes were statistically insignificant [Figure 2b]. The markers associated with oxidative stress were also found to have a transient response up to 3 weeks; thereafter, the response went back toward the basal levels [Figure 2c]. The results inferred that the transcriptional changes at mRNA level induced by drug "9u" were translated into changes at the protein level and to further activity of the cells. There was linearity in the data obtained at the both transcriptional and translational levels, but the changes are transient and statistically non-significant and could be restored by the cells themselves over a period of time during the chronic exposure of the test compound, i.e. 7 weeks.

DISCUSSION

There are a range of drugs such as INH, rifampicin and pyrazinamide, etc., that serve as therapeutic agents for the treatment of TB. But, the major adverse effect associated with all these drugs is their severe hepato and other systemic toxicities.[21-24] Thus, attempts are being made worldwide to develop more effective and less-toxic anti-TB drugs, which can also offer economic viability. In order to address the issue of toxicity-induced adverse effects of existing anti-TB drugs, we have also synthesized a library of symmetrical *trans*-cyclohexane-1,4-diamine and evaluated them against the *M. tuberculosis* H37Rv stain to assess their applicability as potential anti-TB drug candidate molecules. Of the 27 compounds tested, four compounds were found to have significant activity against the *M. tuberculosis* H37Rv stain. Compound "9u" having the i-propyl group substitution at the p-position was found to be the most potent among all the tested compounds.[19] Further, biological safety of the compound "9u" to the host system was evaluated at and higher than the therapeutic doses in human lung epithelium cells-A549.[20] The compound was found to be safe up to many folds higher to the therapeutic doses to be used to kill the pathogen. But, that study was for a shorter duration and represents the acute and sub-acute *in vitro* studies; however, the drugs for TB are to be prescribed for a longer period. Thus, the present investigations were carried out to study whether the anti-*M. tuberculosis* doses of compound "9u" impose any apoptotic/necrotic response in human lung epithelium cells-A549 following a long-term exposure or not. In the present investigations, we recorded a transient but non-significant apoptotic and oxidative stress response of the test compound "9u" in human lung epithelium cells-A549. This transient response was seen to return to the basal level on a long-term exposure of 7 weeks. Contrary to most effective anti-tuberculosis drug combinations, i.e. INH, rifampicin and pyrazinamide have been reported to induce significant toxicity by inducing persistent oxidative stress, cell injury and apoptosis in human beings.[25-28]

In our studies, transient apoptosis/oxidation-induced toxicity with less magnitude of test compound "9u" till only the third week might be attributed to the high metabolic rate of the cells, which either allowed the test compound a less time to stay or formed a less toxic/no toxic secondary metabolite(s) of the principle compound. Besides, it is also speculated that cells became acclimatized to accommodate the test compound by performing additional metabolic activities. While HepG2 cells have the expression of a wide range of phase I enzymes such as cytochrome P450s (CYPs) and phase II enzymes, 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, an important cytochrome P450 enzyme responsible for the metabolism of foreign compounds.[29-34] These metabolized intermediates cause apoptosis/necrosis-induced toxicity in the liver and kidney, which is the second extrahepatic site rich in metabolizing enzymes. Linezolid, a member of the oxazolidinone antibiotic class, was approved in 2000 for drug-resistant, gram-positive bacterial infections.[35] Linezolid was found to have severe toxic responses in a post-marketing survey when given for 28 days' therapy to human subjects.[36,37] The data on long-term use of linezolid have demonstrated a serious nature of neuropathies,[38,39] which could be correlated to the inhibition of mitochondrial protein synthesis.[40,41] Reactive metabolites of mono-acetyl hydrazine (MAH) are also reported to be toxic to the tissues through free radical generation.[42] In rats, the free radical scavenger glutathione-related thiols and antioxidant glutathione peroxidase and catalase activities are diminished by INH, although glutathione reductase activity is increased.[43,44]

However, experiments have not been carried out to study the specific binding of the test compound "9u" with the cytosolic receptor of the cells. These data could be helpful in elaborating our hypothesis toward the exact mechanism(s) of cell - drug interaction and the reasons of no apoptotic responses and oxidative stress in long-term exposure. We could have hypothesized this as *M. tuberculosis* is known to use as many as eight different cell surface receptors in host phagocytic cells and is involved in the survival, replication and pathogenesis of the bacteria.[45,46] On infection, mycobacterium resides within a specialized early phagosomal compartment. Pathogenic mycobacterium prevents fusion with the lysosome, which facilitates evasion of host bactericidal mechanisms and precludes efficient antigen presentation.[47] The major limitation with conventional therapy available today is long treatment period, i.e. a minimum of 6 months. It is a well-known fact that chronic exposure of drugs for such a long period induces toxic responses in the liver, kidney, lungs and other

vascularized organs.[48] Apart from such toxic responses, the MDR in Mycobacterium spp. is another serious issue of concern.[49] In fact, natural products are supposed to have a small window of toxicity and, with this view in mind and with supported literature, rifampin as a natural compound inhibiting the ribonucleic acid polymerase in *M. tuberculosis* is being used as one of the safest and effective anti-TB agents. Although it has comparatively less-reported toxicity than ISN and pyrazinamide, it is certainly known for significant hepatotoxicity.[50,51,52] Therefore, under these circumstances, our findings showing no significant apoptosis, oxidative stress and cell injury and subsequent toxicity in the cells of target organs may be a step toward the development of 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.

In summary, our findings in human lung epithelium cells-A549 identifies that the test compound "9u" is biologically safe even at the concentrations many fold higher than that of the effective concentrations against the activity of *M. tuberculosis* H37Rv stain. However, *in vivo* investigations using experimental models of TB are needed to reach any firm conclusion about the applicability of the test compound "9u" for therapeutic interventions.

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