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Ranolazine as a protective agent against Lung Cancer: A translational approach

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ABSTRACT

Lung cancer is a malignant lung tumor characterized by uncontrolled cell growth. Ranolazine is an anti-anginal drug which shows its effect by inhibiting latent sodium channel. The aim of this study was to evaluate the protective effect of Ranolazine on urethane induced lung cancer in BALB/c mice. *In silico* molecular docking studies and *in vitro* HDAC2 assay was performed against human HDAC2 protein. MTT assay was assessed against A549 & HepG2 cell lines to corroborate, *in vivo* study was done where urethane was administered 0.5 mg/gm i.p twice a week for a period of 4 weeks, Ranolazine 50 mg/kg, 100 mg/kg p.o daily as low and high dose respectively and methotrexate as standard 0.5 mg/kg p.o. toward the end of 5th week various antioxidant parameters like MDA, GSH, CAT, SOD, were estimated and excise lung tissue was subjected to histopathological evaluation. The molecular interactions of ranolazine with HDAC2 enzyme were also supported by molecular docking simulations. *In vitro* MTT revealed higher Cytotoxicity in A549 (IC_{50} 209.43 g/ml) compared to HepG2 (IC_{50} 236.87 g/ml). In the present study on treatment with Ranolazine (50 & 100 mg/kg, p.o) antioxidant marker like MDA, GSH, CAT, SOD was significantly altered. In conclusion present study suggests that treatment with ranolazine has shown protective effect on urethane induced lung cancer in mice.

INTRODUCTION

The most common type of all cancers is lung cancer which is characterized by abnormal and uncontrolled growth of cells. According to world health organization (WHO) there were about 14 million new lung cancer cases reported in 2012 with an expected surge of about 70% within coming two decades. About 85% of lung cancer cases are categorized under non-small cell lung cancer (NSCLC) subtype which has about 17.3% of survival rate for a period of 5 years [1]. In India there is steady growth in lung cancer incidence since 1957 with variety of cancer subtypes encompassing bronchogenic carcinoma, squamous and small cell carcinoma with M:F ratio of 4.5:1 [2-3].

There is a vast array of risk factors associated with higher immergence of lung cancer; the primary reason responsible is believed to be tobacco smoke. Smoking accounts for about 80% and passive smoke approximately 30% of overall cases however occupational exposure to carcinogens like asbestos and radon accounts for lung cancer in nonsmokers [4].Misdiagnosis and inappropriate treatment is also a cause of concern in India leading to delayed prognosis [5]. More over genetic polymorphism in different genes like 5p15.33 & 15q24-25.1 predisposes towards genetic accountability in lung adenocarcinomas [6]. Oxidative stress has a wide spectrum of effects on tumor suppressors leading to development of lung cancer [7]. In addition to it several molecular mechanisms pertaining to the explicit role of voltage sensitive ion channels (Na^{2+}, K^{+}, Ca^{2+}) has also been witnessed in cancer development and metastasis [8].

Ranolazine, a piperazine derivative was approved in the year 2006 and is been safely used clinically ever since, it is been used in alleviating cardiovascular complications like angina and arrhythmias. Researchers postulate that ranolazine shows its activity by inhibition of ^{l}NaL, late rectifying K^{+}& L-type Ca^{2+} channels [9]. Since voltage sensitive ion channel are up regulated in cancer, studies on inhibition of such channels provides a newer target for cancer treatment. Blockade of $Na_{v}1.5$ channel prevents breast cancer invasiveness and lung metastasis [10]. The present study deals with assessing the protective effect of ranolazine on

lung cancer. *In silico* molecular docking study of ranolazine was conducted against human HDAC2 protein and in accordance to it *in vitro* HDAC2 assay was performed. MTT assay was performed against A549 & HepG2 cell lines to corroborate the result study were extended to in vivo using an appropriate murine model.

Well known animal model used in the study of lung tumor genesis is urethane induced cancer, urethane is a carbamate compound found in tobacco smoke and many fermented products. It produces distinct type of tumors which resembles to human lung adenocarcinoma [11]. Urethane is known to produce pro-inflammatory responses which eventually changes cellular micro environment promoting cancer development [12].

MATERIALS AND METHODS

CHEMICALS

Urethane, thiazolyl blue tetrazolium bromide, Ellman's reagent, thio barbituric acid fetal bovine serum, RPMI media was procured from Sigma Aldrich. Methotrexate and ranolazine were generous gift from MSN Laboratories, Hyderabad. FLUOR DE LYS® HDAC flurometric activity assay kit. All the salts and solvents used were of analytical grade.

IN SILICO MOLECULAR DOCKING STUDY

Ligand preparation

The 2D to 3D structure conversion of ranolazine was performed in Maestro 9.5. Using LigPrep (2.5) module, geometry of the drawn ligand was optimized by Optimized Potentials for Liquid Simulations-2005 (OPLS-2005) force field. A total of 10 conformations were generated.

Protein preparation

The co-crystal structure of HDAC2 protein (PDB IDs: 3MAX) was obtained from the RCSB protein data bank (http://www.rcsb.org/pdb). The Multi step Schrödinger's Protein preparation wizard (PPrep) has been used for final preparation of protein. The protein was preprocessed separately by deleting the substrate co-factor and water molecules with in 5Å distance (water without H bonds), addition of missing loops and amino acids in PDB file using PRIME followed by optimization of hydrogen bonds. PPrep neutralizes side chains and residues which are not involved in salt bridges. This step is then followed by restrained minimization using the OPLS 2005 force field to RMSD of 0.3Å. For all calculations Zn^{2+} at active site pocket was retained as it is important for catalytic activity of HDAC's

Docking

After preparation of protein and ranolazine, Glide XP (Extra Precision) docking was initiated at active site of protein using Glide 5.8 module in Maestro 9.5 (Schrödinger suite 2014). Receptor grid was generated at the co-crystal binding site of protein with co-ordinates of X: 66.55, Y: 29.58, Z: 1.35 and length 20Å.

IN VITRO HDAC2 ENZYME ACTIVITY STUDIES

In vitro HDAC2 inhibition was performed using HDAC2 Fluorimetric Drug Discovery Kit. The Fluor de Lys®-Green HDAC2 assay is based on unique HDAC2 substrate/developer combination. The Fluor de Lys® - Green Substrate comprises of an acetylated lysine side chain which was tagged with aminomethylcoumarin (AMC). The fluorescence signal is generated in proportional to the amount of deacetylation of lysine in the substrate. Activity was measured by incubating test compound with 4 ng/well HDAC2 enzyme and substrate. After 30 min incubation, reaction was quenched by adding developer. Deacetylation was measured in terms of fluorophore release at 485 nm excitation and light (470-500 nm) and ~ 530 nm emissions on multiplate reader.

CELL CULTURE

HepG2 (hepatocellular carcinoma), A549 (lung carcinoma) and HEK293 (Human Embryonic Kidney) cell lines were grown in RPMI-1640 supplemented with 10% heat inactivated FBS, 100 IU/ml penicillin, 100 mg/ml streptomycin and 2 mM glutamine. Cultures were maintained in a humidified atmosphere with 5% CO_2 at 37°C. The cultured cells were subcultured twice a week, seeding at a density of about 2×10^3 cells/ml. Cell viability was determined by the trypan blue dye exclusion method.

Cell viability and Counting

Cell viability was determined by the trypan blue dye exclusion method and counted using hemocytometer. Trypan Blue is a vital dye and its reactivity is based on the fact that the chromophore is negatively charged and does not interact with the cell unless the membrane is damaged. Therefore, all the cells which exclude the dye are viable. 0.1 ml of 0.4% trypan Blue Stain was added to $1*10^5$ cells and observed and counted under microscope by filling on a hemocytometer. Cells viable will exclude the dye whereas stained cells represent dead cells [13].

Cell proliferation assay

Antiproliferative/Cytotoxicity effect of test compounds on various cell lines was assessed using the MTT assay as described by Mossman [14]. The MTT assay was based on the reduction of the tetrazolium salt, MTT, by viable cells. The mitochondrial dehydrogenase enzyme converts the yellow form of the MTT salt to insoluble, purple formazan crystals using NADH or NADPH as coenzyme. Briefly, HepG2 and A549 and HEK 293 (5×10^3 cells/well) cells were incubated in 96-well plates in the presence or absence of test compounds (Ranolazine) at different concentrations (0, 0.5, 1, 10, 50,100 and 150, 200, 250 and 300 µg/ml) for 24 hr in a final volume of 200 µl. At the end of the treatment (21st hr), 20 µl of MTT (5 mg/ml in PBS) was added to each well and incubated for an additional 3 hours at 37°C. The purple-blue MTT formazan precipitate was dissolved in 100 µl of DMSO. The activity of the mitochondria, reflecting cellular growth and viability, was evaluated by measuring the optical density at 570 nm on a multi-well plate reader (Victor3TM, Perkin Elmer). Each concentration was tested in three different experiments run in four replicates. Means and standard deviations were calculated and reported as the percentage of growth vs control.

WESTERN BLOT ANALYSIS

Western blot analysis was performed according to procedure described by Ravi et al [15]. Briefly, cells were treated with test compound for 24hrs and lysed in a lysis buffer and centrifuged (10,000×*g*) for 10 min. The protein content was analyzed according to Bradford method (Bradford, 1976). Proteins (100 µg) were separated using 812% sodium dodecyl sulphate-polyacrylamide gels (SDS-PAGE) along with protein weight standards, electrophoretically transferred to nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA). Non specific binding sites on the membranes were blocked with 5% (w/v) nonfat dry milk after checking the transfer using 0.5% Ponceau *S* and probed with a relevant antibody (HDAC2 1:1000 dilution)

and (P53 1:1000 dilution) for 8-12 hr at 4°C followed by detection using peroxidase-conjugated secondary antibodies and Super Signal West Pico Chemiluminescence Substrate (Thermofisher scientific, USA). Equal protein loading was detected by probing the membrane with β-actin antibodies.

INVIVO STUDIES

Experimental animals

BalB/c male mice weighing 17-20 gm were procured from National Institute of Nutrition, Hyderabad. They were housed in clean and transparent polypropylene cages (5 mice/cage) and maintained at standard environmental conditions of room temperature, relative humidity (45%), 12-hr light/dark cycle and had free access to food and water. After 3 days of acclimatization, they were randomly distributed into 5 experimental groups of 6 each with equal group weights. The study was reviewed and approved by the Institutional Animal Ethics Committee, G. Pulla Reddy College of Pharmacy, Hyderabad, India (GPRCP/IAEC/07/15/2/PCL/AE-1A-MICE-M-30).

Experimental protocol

After the lag phase of acclimatization period dosing of the animals was done in the following manner. Group I (Normal control): Received saline for a period of 4 weeks. Group II (Disease control): Received urethane (500 mg/kg i.p) twice a week for a period of 4 weeks. Group III (Disease treated-low dose): Received urethane (500 mg/kg i.p) twice a week for a period of 4 weeks and ranolazine (50 mg/kg p.o) daily for a period of 4 weeks. Group IV (Disease treated-high dose): Received urethane (500 mg/kg i.p) twice a week for a period of 4 weeks and ranolazine (100 mg/kg p.o) daily for a period of 4 weeks. Group V (Standard): Received urethane (500 mg/kg i.p) twice a week for a period of 4 weeks and methotrexate at a dose of 0.5 mg/kg p.o daily for a period of 4 weeks. At the end of 5th week animals were sacrificed and various parameters were analyzed from serum and excised tissues.

Evaluation parameters:

Estimation of Malondialdehyde (MDA) levels in lung tissue

Malondialdehyde is a product of poly unsaturated lipids which are degraded by reactive oxygen species. This is a highly reactive compound that causes toxic stress within the cell and this aldehyde is used as marker to measure the levels of oxidative stress in the cell. The estimation of it is based up on a reaction with thiobarbituric acid forming a colored substance that absorbs strongly at 532 nm. 500 l of 2% lung tissue homogenate in 0.15 mol/L with 200 l of 8% SDS was incubated at room temperature for 5 min, later 1.5 ml of each 20% acetic acid and 0.8% thiobarbituric acid was added and heated at 95ºC for 90 min after cooling 5 ml of butanol and pyridine mixture in a ratio of 15:1 was added followed by subjecting to centrifuge, absorbance of resultant colored layer was measure at 532nm [16].

Determination of superoxide dismutase (SOD)

Superoxide dismutase (SOD) enzymes that alternately catalyze the dismutation of the superoxide (O_2^-) radical into either ordinary molecular oxygen (O_2) or hydrogen peroxide (H_2O_2).The oxidation of epinephrine results into the formation of adrenochrome which exhibits a characteristic absorption at 480nm. Furthermore epinephrine is unstable at a pH 10.2 and results in autoxidation which is strongly inhibited by SOD. 2% of lung tissue homogenate in 0.1 M phosphate buffer was taken to it 1.5 ml of carbonate buffer (pH 10.2), 0.5 ml of EDTA, 0.4 ml of epinephrine was added before taking the absorbance at 480 nm [17].

Determination of catalase (CAT)

The enzyme catalase causes lysis of H_2O_2 into water and oxygen, decrease in absorbance represents consumption of H_2O_2 by the enzyme as a measure of enzyme concentration. 0.1 ml of 2% homogenate in 0.1 M phosphate buffer was taken in a cuvette containing 1.9 ml of 50 mM phosphate buffer (pH7.0), 1 ml of H_2O_2 was then added to initiate the reaction. Absorbance was taken at 15 and 30 seconds at 240 nm [18].

Determination of glutathione levels (GSH)

5,5-dithiobis (2-nitrobenzoic acid) (DTNB) is a chromogen which is reduced by GSH to a yellow colored complex showing an intense absorption at 412 nm. The intensity of absorption is directly proportional to the GSH concentration in the sample. 0.5 ml of tissue homogenate was taken to it equal volumes of TCA (20%) and EDTA was added for protein precipitation and kept on standing for 5 minute, after subjecting it to centrifugation 200 l of supernatant was transferred to tubes containing 1.8 ml of Ellman's reagent (DTNB). Soon after the completion of reaction, solution was read using UV spectrophotometer at 412nm [19].

Histopathological evaluations

Excised lung tissues were fixed in 10% buffered formalin for 24 hr the specimens were dehydrated using ethanol and embedded in paraffin wax. Sections were later deparaffinized using xylene and ethanol. The slides were washed with PBS and permeabilized with 0.1M citrate, 0.1% triton X-100, these sections were stained with hematoxylin and eosin and observed under light microscope.

Statistical analysis

Data are expressed as mean ±SEM for experiments in triplicates. Variances in different groups were calculated by one way ANOVA analysis followed by post tukey's test using Graph Pad Prism.

RESULTS

Molecular docking

Molecular docking calculations revealed that ranolazine is fitting in to the active site pocket of HDAC2 with docking score of -8.343 and showing interactions with important amino acids which are in vicinity of active site pocket of HDAC2 which is evidenced by hydrogen bond formation with amino acids His146, Tyr308 and Asp104. NH+ group of ranolazine showed strong π-cation interaction with amino acid Hie183, Phe155 and Phe210. It also showed interaction with Zn2+ metal at active site pocket which is important for catalytic activity of enzyme. The ligand interaction diagrams of co-crystal ligand and ranolazine are shown in (Figure 1).

HDAC2 assay

At 10 mM concentration, ranolazine was found to exhibit close to 56% enzyme inhibitory activity (Figure 2).

Cell proliferation assay

Anti proliferative effects of Ranolazine on A549 and HepG2 along with normal cell lines was evaluated by MTT assay. A dose-dependent decrease in the growth of both the cell lines was

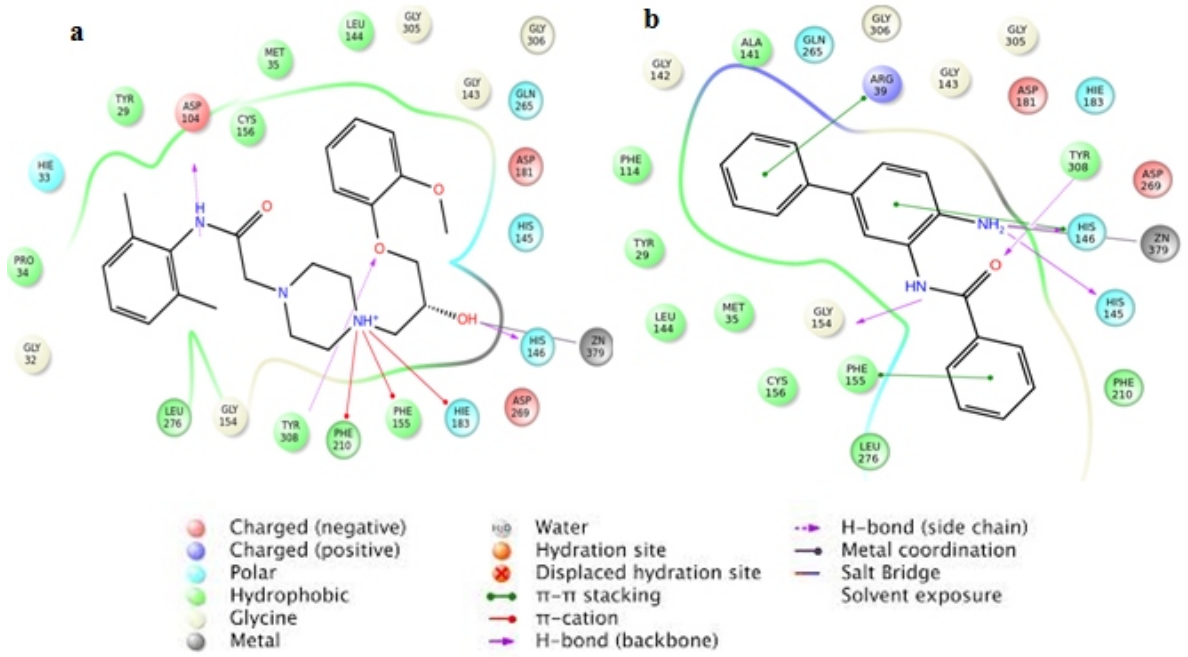

Fig. 1 : Simulated molecular interaction of ranolazine with HDAC2
(a) Ligand interaction diagram of ranolazine. (b) Ligand interaction diagram of co-crystal ligand.

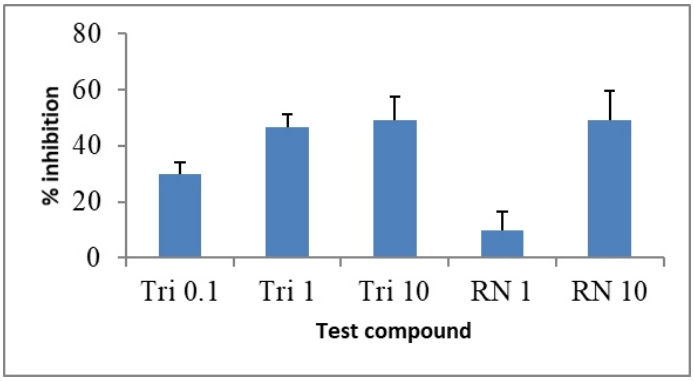

Fig. 2 : *In vitro* HDAC2 assay
Ranolazine showed dose dependent inhibition of HDAC2.

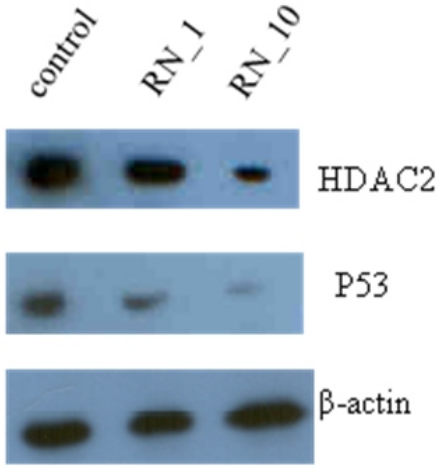

Fig. 4 : Western blot analysis : A dose dependent response of ranolazine against HDAC2 and p53.

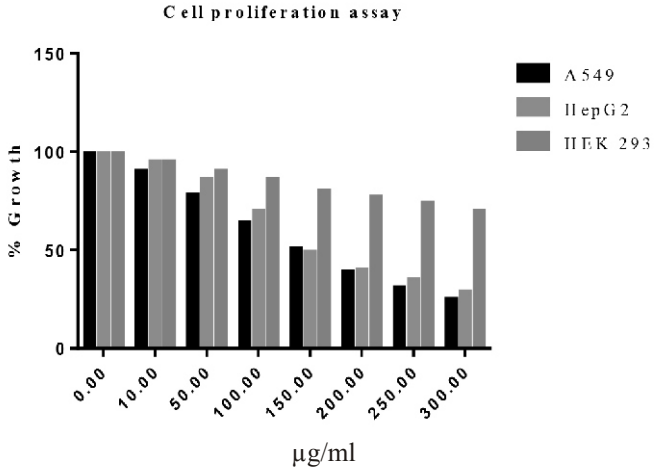

Fig. 3 : Cell proliferation assay
Ranolazine exhibiting cytotoxicity towards A549 cell line and minimal cytotoxicity towards HEK293

observed but Ranolazine showed a better activity on A549 cells with an IC_{50} value 209.43 µg/ml and the IC_{50} value in HepG2 cells was found to be 236.87 µg/ml (Figure 3).

Western blot analysis

A dose dependent decrease in the HDAC2 protein levels were observed by ranolazine. An apoptotic marker P53 levels also decreased dose dependently indicating the P53 mediated HDAC2 inhibition (Figure 4).

Biochemical Estimations

MDA levels

Malonaldehyde is a metabolic product of lipid peroxidation in cells and a byproduct of prostaglandins. Urethane administrations

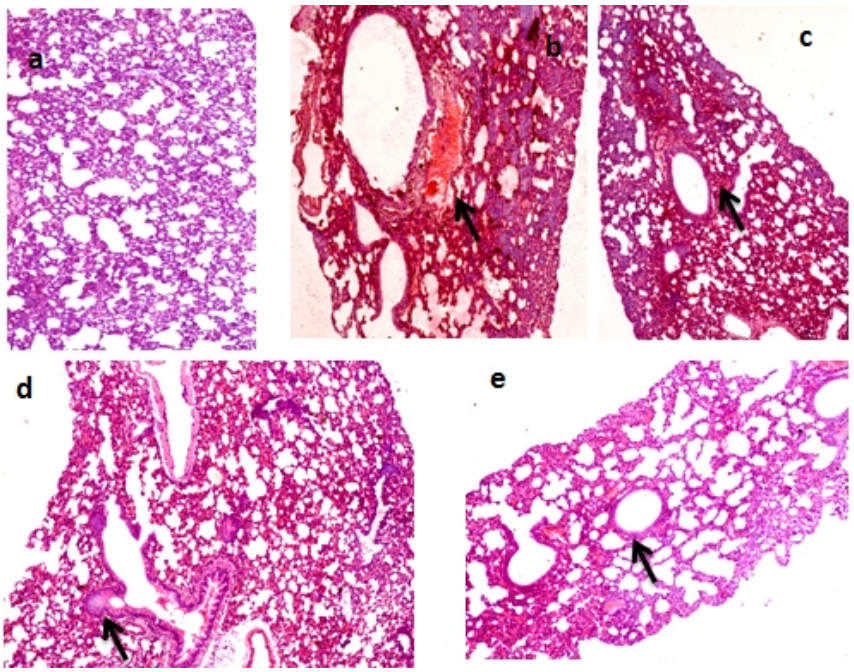

Fig. 5 : Histopathological evaluation

(a) Normal control (b) Disease control 0.5 gm/kg Urethane showing significant signs of inflammation (c) Standard control 0.5 mg/kg methotrexate (d & e) Ranolazine 50 and 100 mg/kg alleviating inflammatory signs and exhibiting protective effect against oxidative damage to lung tissue dose dependently.

Table 1 : Consolidated values of MDA, CAT, SOD and GSH from different groups. Data was expressed as mean ± SEM (n=6). Data analyzed by one way analysis of variance (ANNOVA), followed by Tukey's test for the comparison of means; [a]p<0.001 compared to Normal control group, [c]p<0.001,[b]p<0.01 compared to Disease control group.

Groups	MDA (nmol/mg)	SOD (µmol/mg)	Catalase (K/ml)	GSH (µmol/gm)
Normal control	7.60±0.47	0.53±0.025	0.268±0.021	6.468±0.217
Disease control	17.27±0.47[a]	0.08±0.012[a]	0.038±0.003[a]	2.892±0.230[a]
Standard control	10.49±0.53	0.34±0.004	0.075±0.01	5.400±0.181
Ranolazine(50mg/kg)	12.96±0.31[b]	0.39±0.021[b]	0.177±0.004	3.752±0.176[b]
Ranolazine(100mg/kg)	9.98±0.37[c]	0.46±0.029[c]	0.221±0.005	5.723±0.268[c]

produced significant increase in MDA levels when compared to control. Marked reduction of MDA levels was observed in ranolazine treated groups dose dependently (Table 1).

SOD level

Superoxide dismutase is a type of metalloenzyme which counter acts to the harmful effects if super oxides produced by the cell in metabolic processes. Decline in the SOD enzyme levels was observed in diseased group and significant increase was observed with in treated groups producing near to normal levels (Table 1).

Catalase levels

It is a ubiquitous enzyme present in most of the cell which helps to catalyze metabolically generated H_2O_2 into water and oxygen. Reduction in enzyme activity was seen in diseased group on the other hand notable increase was observed in dose dependent manner with in treated groups (Table 1).

GSH levels

Glutathione is an antioxidant endogenous to the cells significant decrease in glutathione was evident in diseased group.

The decline was compensated in treated groups dose dependently near to normal at a concentration of 100 mg/kg body weight . The consolidated values of MDA, CAT, SOD and GSH were represented in Table 1.

Histopathology

Administration of urethane in disease control group had produced remarkable pulmonary damage causing inflammation as seen in Figure 5b and the damage was countered due to concurrent administration of methotrexate 0.5 mg/kg, ranolazine 50 mg/kg, 100 mg/kg to respective groups as seen in Figure 5c 5e.

DISCUSSION

Oxidative damage to the cell can be of two origins extracellular and intracellular, where extracellular damage to cell is produced by variety of environmental factors and intracellular damage can be due to metabolic activities and inflammation. This eventually leads to DNA damage causing mutations and alterations in repair mechanism [20]. There is a well established link between chronic inflammation, development and progression of cancers originating from lung, colon and liver. Since inflammation produces oxidative stress and different growth stimulus like angiogenesis promoting cancer invasion and metastasis [21] [22]. A similar type of relation exist in development of lung cancer in human beings as chronic smoking leaves the person vulnerable to inflammatory disorders like emphysema and chronic obstructive lung disease causing epigenetic changes and eventually leading to cancer, such type of prognosis is commonly found in most of the lung cancer cases [23]. Efforts are been done to investigate newer targets like HDAC family proteins which are involved in epigenetic control of various nuclear events [24]. Studies has shown significant role of HDAC2 in cell cycle regulation and apoptosis and over expression in lung cancer [25]. Molecular docking studies of Ranolazine on human HDAC2 protein revealed prominent binding affinity with strong H-bond and π-cation interactions. Significant HDAC2 inhibitory action of 56% and protein levels of HADC2, p53 demonstrates induction of apoptosis by ranolazine.

Urethane causes lung cancer by producing inflammation which resembles emphysema and COPD in humans resembling to the adenocarcinomas [26]. To study the protective effect of ranolazine against lung cancer urethane served as an appropriate model inducer. The *In vitro* MTT assay shows the inhibition of cancerous cell growth and on the contrary was found to be neutral in normal cells; this shows the selectivity of ranolazine to the cancerous cells and such an effect may be due to over expression of ion channels in transformed cells [27]. As observed from in vivo studies, daily administration of ranolazine effectively reduced urethane induced oxidative stress represented by MDA (9.98 ± 0.37 nmol/mg) which was near to the normal value (7.60 ± 0.47 nmol/mg) and prevents the cell from damage. Other antioxidant parameters like SOD, CAT, GSH was also reduced to 0.46 ± 0.029 mol/mg; 0.221 ± 0.005 k/ml; 5.723 ± 0.268 mol/gm respectively suppressing the oxidative stress. Histopathological evaluation of lung tissue exhibits significant inflammatory signs in disease control group where as ranolazine low and high dose showcased remarkable protection against lung damage due to oxidative stress. The recent researches are focused towards marketed compounds which act by inhibiting ion channels there by reducing the expense of discovering a novel compound, since the safety profile of such compounds will be pre-established [28]. With the use of antineoplastic drugs serious adverse effects are associated such as thrombocytopenia, leucopenia, bone marrow depression, alopecia, erythema etc, more over risk of secondary cancer rises [29]. Ranolazine being safe does not possess any such serious adverse effects and can be effectively used to prevent cancer invasiveness. Targeted drug delivery system and modified dosage form can increase the efficacy by preventing unwanted localization of the drug. Further studies are required to establish the molecular mechanism involved in prevention of cancer invasiveness and metastasis by Ranolazine.

CONCLUSION

The present work demonstrated anti proliferative effects of ranolazine on A549 cells with an IC_{50} value of 203.49 µg/ml and significant HDAC2 inhibition. Ranolazine effectively alleviates the oxidative stress induced by the urethane in mice. Thus, As evident from the insilico, *invitro* and *invivo* studies we propose that ranolazine may be used as safe and effective drug for the management of lung cancer.

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