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LACTATE TRANSPORT AND ACIDOSIS AS TARGET FOR TUMOR REGRESSION BY DRUG REPOSITIONING IN LUNG CARCINOGENESIS MODEL

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ABSTRACT

Objective: The objective of the present study was to inhibit the lactate transport and acidosis by using atorvastatin, pantoprazole and sodium bicarbonate in urethane induced lung carcinogenesis model **Method**: The day of starting the experiment, the animals were divided in to 5 groups for six animals each. The group I serve as control and group II serves as negative control receive 600mg/kg urethane and group III, group IV and group V serve as treatment groups receive atorvastatin (10mg/kg), pantoprazole (20mg/kg) and sodium bicarbonate (200mmol/L) respectively from the day of urethane administration once daily orally for 26 weeks. . During the period of study the body weight of the animal were recorded. At the end of the study blood samples are collected by retro orbital puncture and evaluate the tumor marker, inflammatory marker, blood lactate pyruvate LDH and glucose. After blood collection, the lung immediately excised and washed with ice cold saline and weighed to calculated the relative lung weight and lung tissue were used to evaluate the tissue lactate, pyruvate and SDH. The portion of the lung tissue was subjected to histopathological examination to evaluate the microscopical changes in the tissue. **Result**: In this present study, groups of animal treated with atorvastatin, pantoprazole and sodium bicarbonate showed a significant decrease in cancer incidence with decrease in tumor load, tumor volume and multiplicity compared with cancer control. The markers of glycolytic stress were significantly decreased and SDH levels were significantly increased compared to cancer control. The histpathology of lung supported

the biochemical parameters, showed inflammatory infiltrates without any incidence of neoplastic cells. **Conclusion**: From the result, it was observed that atorvastatin, pantoprazole and sodium bicarbonate showed a significant protection against urethane induced lung carcinogenesis. This might be due to inhibition of mono-carboxylate transporters and metabolic acidosis in cancer cells. These finding provide new evidence for the mechanism by which this class of drugs may be acting on cancer cells.

KEYWORDS: Anti-cancer activity, Urethane, Atorvastatin, Pantoprazole, Sodium bicarbonate, Mono-carboxylate transporter.

1. INTRODUCTION

Cancer can be defined as an un-regulated growth of cells arising from one cell. Lung cancer is a type of cancer characterized by uncontrolled cell growth in tissue of lung.[1,2,3] Killing of cancer cells without significant toxicity to normal cells is one of the most important considerations in cancer chemotherapy. Understanding the biological difference between cancer cells and normal cell is essential for design and development of anti-cancer drug with selective anticancer activity. Cancerous cell even in the presence of oxygen convert glucose to lactate by an altered energy metabolic pathway less efficient than oxidative phosphorylation. This along with increased degradation of glutamine leads to metabolic acidosis.[4,5]

The end product of glycolysis, lactate is produced in large excess in tumors. The active secretion of lactic acid outside the tumor cell significantly contribute to the acidification of the extracellular mileu, In addition to other mechanism promoting tumor acidosis. This renders the environment around the tumor tissue more suitable for colonization and invasion by cancer cells. Moreover lactate also actively stimulates tumor cell migration y activation of β1 integrin and angiogenesis following a stimulation of VEGF production in endothelial cells.[21-23] Furthermore extracellular lactic acid was found to inhibit the ability of the immune system to eradicate aberrant cells, thus contributing to the immune escape phenomenon. Inhibition of lactic acid generation of caner may be lead to inhibition of proliferation of cancerous cells by dual pathway via energy depletion and by reduction of their immune-suppressive activity in the tumor micro- environment.[6,7,8,9]

In present study, drug repositioning that take advantages of the existing data of pharmacokinetics was focused to identify a new target site[11]. The three existing drug

molecules were selected based on in-vitro studies for evaluation against urethane induced lung carcinogenesis.[12,13] Atorvastatin is a HMG-CoA reductase inhibitor for the treatment of cholesterol and pantoprazole is used for the treatment of acidity.[14,15,16] Atorvastatin showed cytotoxic effect in different malignant cell in-vitro. Recently atorvastatin showed a potent mono-carboxylate transport inhibitory activity in-vivo. Pantoprazole based on preclinical evidence in cancer in in-vitro and in-vivo and recent studies on acidosis inhibition was selected for the study. A strong link between lactate transport and lung carcinogenesis was recently found in urethane induced lung carcinogenesis, so urethane induced lung carcinogenesis is selected for evaluation.[17,18,19,20] The urethane is geno-toxic chemical carcinogen that specifically promote the development of lung cancer, these metabolites causes oxidative stress DNA molecules, causing the development of adducts and c-hydroxylation to form vinyl carbamate which is then converted to an epoxide that interact with nucleic acid, the resulting reactive oxygen species and reactive nitrogen species plays an important role in the initiation, promotion and progression phases of lung cancer.[10, 11] The aim of the study is target lactate transporters and metabolic acidosis by using atorvastatin, pantoprazole and sodium bicarbonate in urethane induced lung carcinogenesis model.

2. MATERIALS AND METHODS

2.1. Chemical and reagent: Atorvastatin, Pantoprazole, Urethane, Carboxy methyl cellulose, Sodium chloride, Crystalline lactic dehydrogenase, Nicotinamide adenine di nucleotide, Sodium bicarbonate, Glycine, Hydrazine hydrate, Sodium hydroxide, Tris-(hydroxyl methyl) amino methane, Concentrated sulphuric acid, Sodium pyruvate, Lactic acid, 2,4-dinitro phenyl hydrazine, Concentrated hydrochloric acid, Dibasic sodium phosphate, Mono basic sodium phosphate, Bovine serum albumin, Succinate, Pottassium ferricyanide, Pottasium hydroxide, Perchloric acid, Glucose reagent, Glucose standard.

2.2. Animals: The experimental protocol was approved by the Institutional Animal Ethical Committee (IAEC) [Proposed no: DAMCOP/IAEC/026] of Devaki Amma Memorial College of Pharmacy, Chelembra, Malappuram, Kerala, India. Male Balb/c mice weighing 25-35 g were used in this study. The animals were procured from Central lab animal facility, Amrita Institute of Medical Science and Research Centre, Ponekkara, Kochi. The animals were housed in propylene cages and were acclimated to laboratory conditions 25 ±2°C with 12h light and dark cycle. They were fed with standard pellet diet and had free access to water.

The study was conducted according to the guidelines for the use and care of experimental animals.

2.3. Experimental design(Urethane induced lung carcinogenesis model)

On the day of the experiment, the animals were divided randomly into control and experimental groups (n=6).

Group I serve as control and receive 0.5% w/v carboxy methyl cellulose suspension 1 ml/100 g once daily orally for a period of 26 weeks.

Group II serve as negative control and receive urethane 600 mg/kg dissolved in sterile 0.9% w/v of Nacl intraperitoneally once weekly for 10 weeks. Following the first injection of urethane the mice received the 0.5% w/v carboxy methyl cellulose 1 ml/100g once daily orally for a period of 26 weeks.

Group III serve as treatment group and receive urethane 600 mg/kg dissolved in sterile 0.9% w/v of Nacl intraperitoneally once weekly for 10 weeks. Following the first injection of urethane the mice received the Atorvastatin 10 mg/kg body weight in 0.5% w/v carboxy methyl cellulose once daily orally for a period of 26 weeks.

Group IV serve as treatment group and receive urethane 600 mg/kg dissolved in sterile 0.9% w/v of Nacl intraperitoneally once weekly for 10 weeks. Following the first injection of urethane the mice received the Pantoprazole 20 mg/kg body weight suspended in 0.5% w/v carboxy methyl cellulose once daily orally for a period of 26 weeks.

Group V serve as standard group receive sodium bicarbonate 200mmol/L in drinking water daily for a period of 26 weeks.

During the period of study the body weight of animals were recorded once weekly till the end of the study. At the end of the study blood samples was collected by retero orbital puncture under anaesthesia (Ketamine 100 mg/kg i.p) and evaluated for tumor marker (CEA-carcino embryonic antigen).

After blood collection the animals was euthanized and the lung immediately excised, washed with ice cold saline, dried using a whatman's filter paper and weighed to calculated the relative lung weight.

Gross morphological examination was carried out to observe lung tumor incidence, multiplicity of tumors and tumor load (sum of the tumor per lung in average).

The lung tissue was subjected to evaluation of inflammatory markers (CRP- reactive protein) and subjected to evaluation of glycolytic stress (Lung lactate, Lung pyruvate, Lung LDH, Lung SDH) A portion of lung tissue was subjected to histopathological examination to evaluated the microscopical changes in the tissue.[24]

2.4. Preparation of blood sample

To 0.5 ml of blood added 2 ml of perchloric acid in an eppendrof tube. Stand for 10 minutes (room temperature) and micro centrifuged for 15 minutes at 3000 rpm. After centrifugation supernatant collected using syringe and kept in freezer (-20°C).

2.5. Preparation of tissue sample: 100 mg lung tissue was homogenized in 2 ml of 6% v/v perchloric acid. The homogenate centrifuged at 5000 rpm for 10 minutes. The supernatant was withdrawn and neutralized with 2 ml 5M potassium hydroxide. Again centrifuged and the supernatant taken for analysis which is kept in freezer (-20°C).

2.6. Estimation of biochemical parameters

2.6.1. Determination of CEA-carcino embryonic antigen

The carcino embryonic antigen is a tumor marker. The concentration of tumor marker in the blood sample were measured by a chemiluminescent immunoassay by using autoanalyzer.[25]

2.6.2. Determination of CRP- reactive protein

The C-reactive protein is inflammatory marker. The CRP level in blood sample was measured by a high-sensitivity immunoturbidimetry method by using autoanalyzer.[26]

2.6.3. Determination of blood glucose

Principle: The glucose present in the blood was determined by glucose oxidase peroxidise (GOD-POD) method. Glucose is oxidised by enzyme glucose oxidase to gluconic acid and hydrogen peroxidase. Hydrogen peroxide further reacts with phenol and 4-aminoantipyrine by the catalytic action of peroxidase to form a red coloured quinoneimine dye complex. Intensity of the colour formed is directly proportional to the amount of glucose present in the sample.[100, 101]

$$\text{Glucose} + 0_2 + H_2O \xrightarrow{\text{GOD}} \text{Gluconic acid} + H_2O_2$$

$$2H_2O_2 + \text{4- amino antipyrene} + \text{phenol} \xrightarrow{\text{POD}} \text{Red Quinoneimine dye} + H_2O$$

Procedure

Labeled the test tubes as Reagent Blank (B), Standard (S) and Test (T). Pipette out 1ml of glucose reagent to all the test tube and added 0.01 ml of distilled water, glucose standard and sample to the Blank (B), Standard(S) and Test (T) respectively. Mix well and incubate at 37°c for 10min or at room temperature (25°c) for 30 minutes. Measure the absorbance of standard and test sample against the blank within 60 minutes. The result was calculated by using the following formula.[27]

$$\text{Total Glucose in mg/dl} \xrightarrow[\text{Abs. S}]{\text{Abs. T}} = \text{X} \quad 100$$

2.6.4. Determination of Lactate

Principle

Lactate was oxidized to pyruvate by the lactate dehydrogenase (LDH) reaction. The hydrazine destroys the pyruvate, allowing the reaction to run to the complete oxidation of all lactate molecules. To ensure this, NAD+ is provided in excess. The concentration of lactate in the sample is proportional to the increase in absorbance as NAD+ is reduced to NADH.

$$\text{Lactate} + NAD^{+} \xrightarrow{\text{LDH}} \text{Pyruvate} + \text{NADH}$$

Procedure

Prepared Reaction Mixture-Reconstitute the appropriate number of NAD vials (Catalog Number N8285) required by pipetting the following reagents into each vial: 2.0 ml Glycine buffer (Catalog Number G5418), 4.0 ml water, 0.1 ml L-Lactate dehydrogenase (Catalog Number L3916). Cap and invert the vials several times to dissolve the NAD. The Reaction Mixture remains active for 4 hours at room temperature or 24 hours at 2–8°C. Pipette into clean dry test tubes labelled as Blank (B) and Test (T). Added 2.9 ml of reaction mixture in both test tube added 0.1ml water, 8% v/v Perchloro acetic acid in to Blank. Added 0.1 ml of biological sample in to Test and mix both gently by inversion. Incubate test tubes for 15 minutes at 37°C and measure the absorbance of Test Sample against the Blank as reference at 340nm.[28]

2.6.5. Determination of Pyruvate

Principle: The pyruvate present in blood and tissue sample reacted with 2, 4, dinitro phenyl hydrazine in the presence of sodium hydroxide to form brown coloured complex, which is determined by spectrophotometrically at 540nm.

Pyruvate+2,4,DNPH $\xrightarrow{\text{NaoH}}$ 2,4 Dinitrophenyl hydrazone (brown color complex).

Procedure: Labelled the test tubes as Reagent Blank (B), Standard(S) and Test (T). 1 ml of the biological sample was taken and to it 1 ml of 0.001 M 2,4- Dinitro phenyl hydrazine and 3 ml of 0.4 N sodium hydroxide to test sample. After 10 minutes, the optical density of the colour was measured in a spectrophotometer at 540 nm against the reagent blank. Pyruvate standards were prepared for comparison.[28,29,30]

2.6.6. Determination of SDH

Principle: The enzyme succinate dehydrogenase catalyses the oxidation of succinate to fumarate in the presence of potassium ferricyanide. The increased concentration of the product (fumigate) is measured spectrophotometrically at 420nm. The activity was expressed as nano moles of succinate oxidized per minute per mg of protein.

Procedure: The reaction mixture containing 1 ml of 0.3 M of phosphate buffer pH(7.4), 0.1 ml of EDTA (0.03 M), 0.1 ml of 3% w/v BSA, 0.3 ml of 0.4 M sodium succinate pH(7.6), 0.2 ml 0.075 M potassium ferricyanide and made up to 2.8 ml with distilled water. The reaction was started by addition of 0.2 ml of mitochondrial suspension. The change in absorbance was recorded at 30 second interval for 3 minutes at 420 nm. The activity was expressed as nano moles of succinate oxidised per minute per mg of protein.[31,33]

2.6.7. LDH Determination

Principle: The enzyme lactate dehydrogenase catalyses the conversion of lactate in the buffered substrate into pyruvate in the presence of NAD. The increased concentration of the product (pyruvate) is measured calorimetrically using 2,4-dinitro phenyl hydrazine. The latter reacts with the pyruvate and gives a brown colour in alkaline medium which is proportional to the amount of pyruvate present in the reaction mixture.

Procedure: Diluted the serum five-fold with saline by mixing 0.2 ml of serum with 0.8 ml of normal saline.

Determination of absorbance of the Blank and Test

Label the test tubes as Reagent Blank(B), and Test(T). Pipette 1 ml of buffered substrate and 0.2 ml of NAD solution in both tubes and placed them in a water bath (37◦C) for five minutes in order to equilibrate the temperature. Added 0.1 ml of serum in the tube marked “T and added 1 ml of serum in the tube marked B (serum blank). Exactly after 15 minutes of

incubation at 37◦C added 1 ml of hydrazine solution to both the tubes. Mixed the contents of each tube and continued incubation for another 15 minutes (exact timing is not necessary). Added 10 ml of NaOH solution (0.4 M) to both the tubes, and mixed. Measure absorbance of Test Sample (Abs.T) and Blank (Abs.B) against water after 10 mins at 505 to 510 nm (green filter). The result was calculated by using the following formula.

$$\blacktriangle A = Abs.T - Abs.B.$$

Determination of absorbance of the standard: Label the test tubes as Reagent Blank (B), and Standard (S) mixed 1 ml of substrate and 0.3 ml of water in a blank test tube. In standard test tube was mixed 1 ml of substrate, 0.1 ml of pyruvate standard and 0.2 ml of water. Added 1 ml of hydrazine solution in both tubes, left for 15 minutes at 37◦C and then added 10 ml of NaOH (0.4N). The absorbance was determined the standard against the blank.[32]

2.7 Determination of relative lung weight: After sacrificing the animals, lung was removed and the washed free of extraneous material and weighed. The relative lung weight was calculated as per the formula given below.

$$\text{Relative lung weight} = \frac{\text{Lung weight}}{\text{Animal body weight.}} \times 100$$

2.8 Morphological examination

Multiplicity of tumor: It is defined as average number of tumors per mouse. And it is obtained by dividing the total number of tumor by the total number of mice per group including non tumor bearing mice.

Tumor volume

$$\text{Tumor volume (v)} = \frac{\prod}{6} \times \text{length} \times \text{width} \times \text{height}$$

Tumor load

$$\text{Tumor load} = \text{Mean number of tumors} \times \text{Mean tumor volume}$$

Tumor incidence

$$\text{Tumor incidence} = \frac{\text{Number of effected mice}}{\text{Total no of mice available for examination}} \times 100$$

2.9. Histopathology of lung: Histopathology is the microscopical study of tissues for pathological alterations. This involves collection of morbid tissues from biopsy or necropsy, fixation, preparation of sections, staining and microscopical examination.

A. ***Collection of materials:*** Thin pieces of 3 to 5 mm, thickness are collected from tissues showing gross morbid changes along with normal tissue.

B. Fixation

Keeping the tissue in Fixative (10% Formalin) for 24-48 hours at room temperature

a) Serves to harden the tissues by coagulating the cell protein,
b) Prevents autolysis,
c) Preserves the structure of the tissue
d) Prevents shrinkage

A. ***Haematoxylin and eosin method of staining:*** Deparaffinise the section by xylol 5 to 10 minutes and remove xylol by absolute alcohol, then wash in tap water. Stain with haematoxylin for 3-4 minutes and wash in tap water. Allow the sections in tap water 5-10 min and wash in tap water. Counter stain with 0.5% haematoxylin and eosin until section appears light pink (15 to 30 seconds), then wash in tap water. Blot and dehydrate in alcohol. Clear with xylol (15 to 30 seconds). Mount in Canada balsam or DPX Moutant. Keep slide dry and remove air bubbles. All the sections of the tissues were examined under microscope for the analyzing the altered architecture due to the urethane challenge and improved lung architecture due to sodium oxamate and 5-bromo isatin. These were examined under the microscope for histopathological changes such as congestion, haemorrhage, neoplasm cells, necrosis, inflammation, Infiltration, and photographs were taken.

2.10. Statistical analysis: The data were expressed as mean ± standard error of the mean (SEM) by instat softwere. Different groups were assessed by one-way analysis of variance (ANOVA) for multiple comparisons followed by Tukey test (Graph pad software Inc, La Jolla, CA. Trial version 7.03).

3. RESULT AND DISCUSSION

Urethane induced lung carcinogenesis model (Body weight of animals)

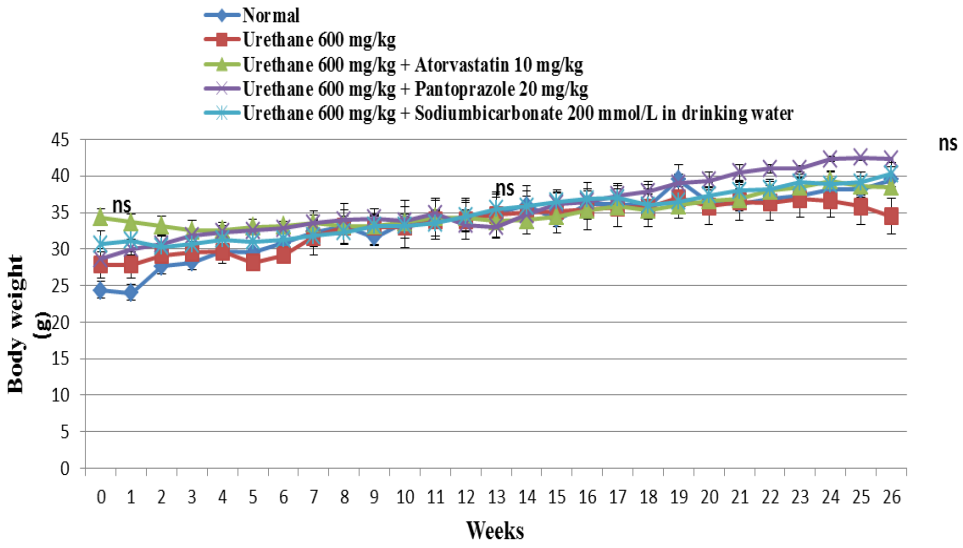

Figure. 1: Effect of Atorvastatin, Pantoprazole and Sodium bicarbonate on body weight in urethane induced carcinogenesis in BALB/c mice.

Values are significantly different from group II urethane induced lung carcinoma, ns- non significant, P values: * p< 0.05, ** p< 0.01,

*** p< 0.001 (one way ANOVA followed by Tukey's test).

Table. 1: Effect of Atorvastatin, Pantoprazole, Sodium bicarbonate on serum CEA and CRP level in urethane induced lung carcinogenesis in BALB/c mice.

Group	Treatment	CEA (ng/ml)	CRP (mg/L)
Group I	0.5 % CMC 1ml/100 g oral	2±0.05774	1±0.09121
Group II	Urethane (URE) 600 mg/kg i.p.	7.65±0.2579***	7.3933±0.302***
Group III	URE 600 mg/kg/ i.p.+ Atorvastatin 10mg/kg oral	2.7866±0.1073***	1.9383±0.03005***
Group IV	URE 600 mg/kg i.p+ Pantoprazole 20mg/kg oral	3.065±0.1662***	1.85±0.05342***
Group V	URE 600 mg/kg i.p+ Sodium bicarbonate 200mmol/L in drinking water	2.845±0.09120***	1.7733±0.0757***

Values are given in Mean±SEM for six animal each.***p<0.001 denotes When cancer control was compared with the normal control and treated group were compared with the cancer control.

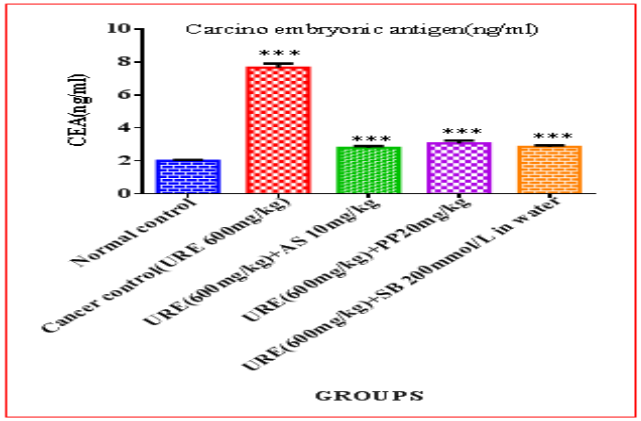

Figure 2: CEA variation observed in different group of BALB/c mice administered with atorvastatin, pantoprazole and sodium bicarbonate.

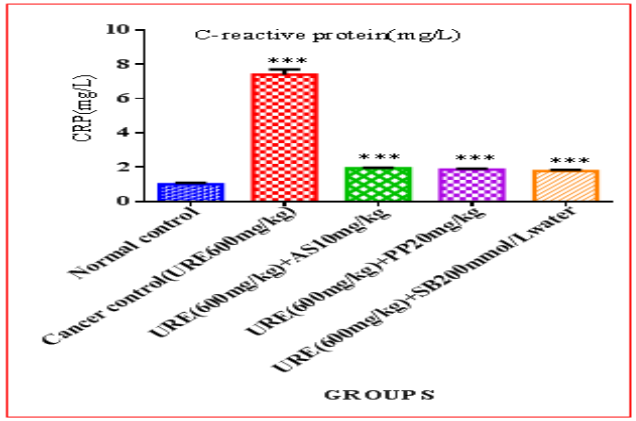

Figure 3: CRP variation observed in different group of BALB/c mice administered with atorvastatin, pantoprazole and sodium bicarbonate.

Table. 2: Effect of Atorvastatin, Pantoprazole and Sodium bicarbonate on tissue lactate, pyruvate, LDH and SDH levels in urethane induced lung carcinogenesis in BALB/c mice.

Group	Treatment	Lactate (µmol/gm)	Pyruvate (µmol/gm)	LDH (nmole)	SDH (nmole)
Group I	0.5 % CMC 1ml/100 g oral	1.52±0.05033	0.0476±0.002789	266.463±5.987	114±2.582
Group II	Urethane (URE) 600 mg/kg i.p.	7.926±0.09465***	0.15583±0.00514***	630.896±6.638***	60.16±2.257***
Group III	URE 600 mg/kg/ i.p.+ Atorvastatin 10mg/kg oral	3.6883±0.1513***	0.08516±0.00414***	389.4916±5.876***	82.8±1.60***
Group IV	URE 600 mg/kg i.p + Pantoprazole 20mg/kg oral	3.9166±0.0358***	0.08883±0.00283***	397.136±4.911***	83±1.98***
Group V	URE 600 mg/kg i.p +Sodium bicarbonate 200mmol/L in drinking water	3.165±0.0452***	0.0833±0.00229***	344.62±5.050***	81.83±2.08***

Values are given in Mean±SEM for six animal each.***p<0.001 denotes When cancer control was compared with the normal control and treated group were compared with the cancer control.

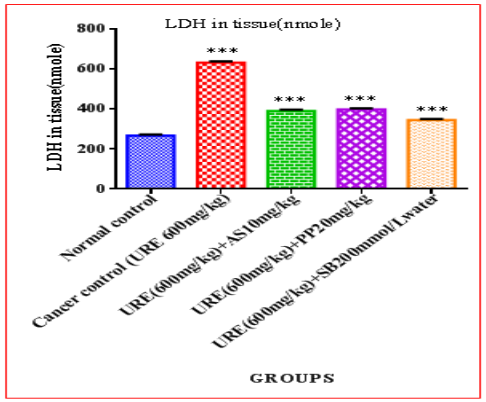

Figure 4: LDH variation observed in different group of BALB/c mice administered with atorvastatin, pantoprazole and sodium bicarbonate.

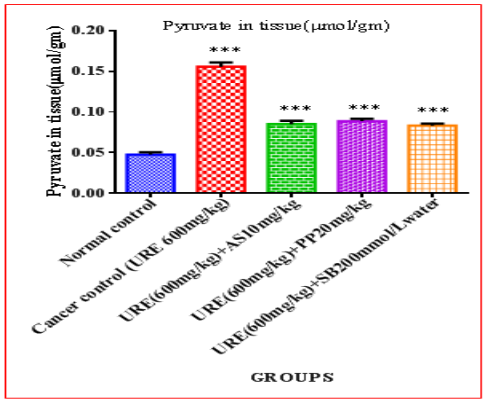

Figure 5: Pyruvate variation observed in different group of BALB/c mice administered with atorvastatin, pantoprazole and sodium bicarbonate.

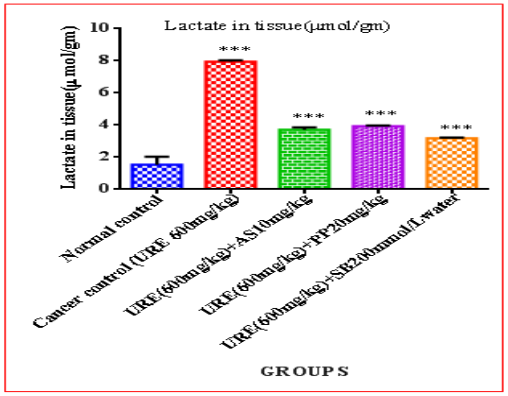

Figure 6: Lactate variation observed in different group of BALB/C mice administered with atorvastatin, pantoprazole and sodium bicarbonate.

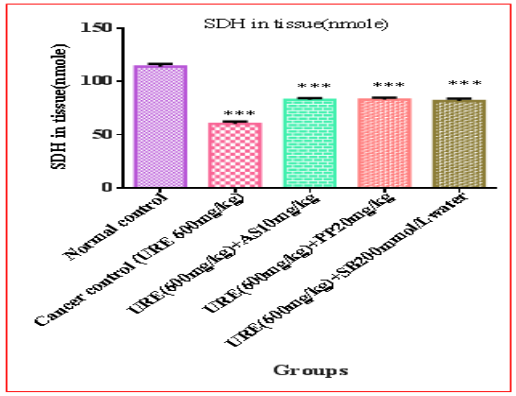

Figure 7: SDH variation observed in different group of BALB/c mice administered with atorvastatin, pantoprazole and sodium bicarbonate.

Table. 3: Effect of Atorvastatin, Pantoprazole and Sodium bicarbonate on blood glucose, serum lactate, pyruvate and LDH levels in urethane induced lung carcinogenesis in BALB/c mice.

Group	Treatment	Glucose (mg/dl)	Lactate (mmole/L)	Pyruvate (mmole/L)	LDH (U/L)
Group I	0.5 % CMC 1ml/100 g oral	74.166 ± 1.376	2.3866±0.05795	0.0425±0.00183	266.843±3.051
Group II	Urethane (URE) 600 mg/kg i.p.	128.5 ± 4.745***	5.1966±0.1897***	0.123±0.007457***	673.67±7.235***
Group III	URE 600 mg/kg/ i.p. + Atorvastatin 10mg/kg oral	91.333 ± 1.145***	3.415±0.1462***	0.0756±0.002076***	384.283±8.696***
Group IV	URE 600 mg/kg i.p + Pantoprazole 20mg/kg oral	87.5 ± 1.118***	3.425±0.1558***	0.08316±0.002613***	357.45±10.457***
Group V	URE 600 mg/kg i.p + Sodium bicarbonate 200mmol/L in drinking water	81.666 ± 2.459***	3.0466±0.04394***	0.07083±0.002301***	345.58±10.86***

Values are given in Mean±SEM for groups of six animals each.***P<0.001 denotes When cancer control was compared with the normal control and treated groups were compared with the cancer control.

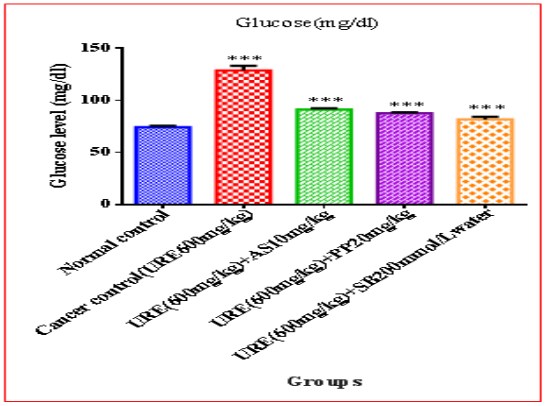

Figure 8: Glucose variation observed in different group of BALB/c mice administered with atorvastatin, pantoprazole and sodium bicarbonate.

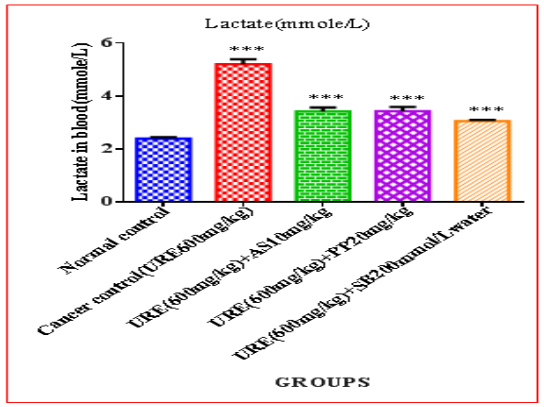

Figure 9: Lactate variation observed in different group of BALB/c mice administered with atorvastatin, pantoprazole and sodium bicarbonate.

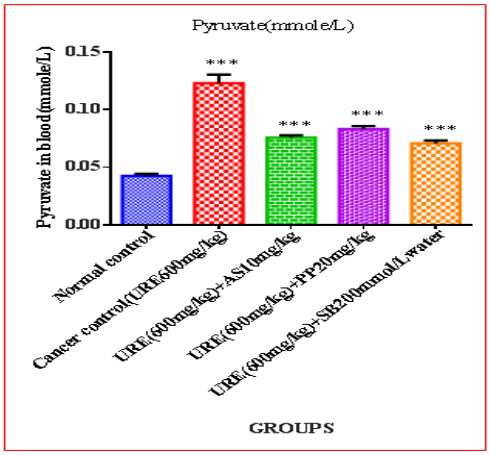

Figure 10: Pyruvate variation observed in different group of BALB/c mice administered with atorvastatin, pantoprazole and sodium bicarbonate.

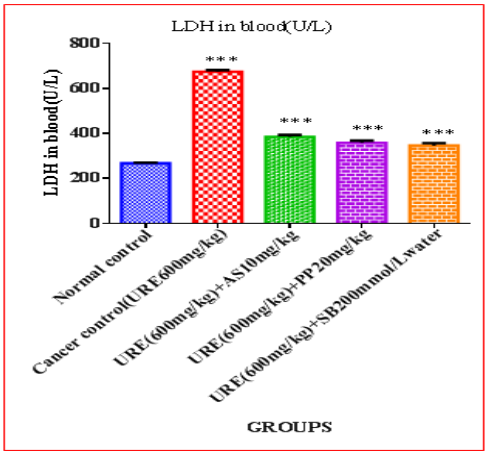

Figure 11: LDH variation observed in different group of BALB/c mice administered with atorvastatin, pantoprazole and sodium bicarbonate.

Table. 4: Effect of Atorvastatin, Pantoprazole and Sodium bicarbonate on tumor incidence, tumor volume, tumor multiplicity and tumor load in urethane induced lung carcinogenesis in BALB/c mice.

Group	Treatment	Tumor incidence	Tumor volume (mm^3)	Tumor multiplicity	Tumor load (mm^3)
Group I	0.5 % CMC 1ml/100 g oral		–	–	–
Group II	Urethane (URE) 600 mg/kg i.p.	100	0.495±0.023***	67.3±7.948***	33.31±0.1842***
Group III	URE 600 mg/kg/ i.p.+ Atorvastatin 10mg/kg oral	60	0.316±0.0108***	38.16±1.956***	12.05±0.021***
Group IV	URE 600 mg/kg i.p+ Pantoprazole 20mg/kg oral	58	0.316±0.0128***	38.66±1.892***	12.21±0.024***
Group V	URE 600 mg/kg i.p+ Sodium bicarbonate 200mmol/L in drinking water	55	0.0305±0.0111***	35.66±1.202***	10.87±0.013***

Values are given in Mean±SEM for groups of six animals each.***P<0.001 denotes When treated groups was compared with the cancer control.

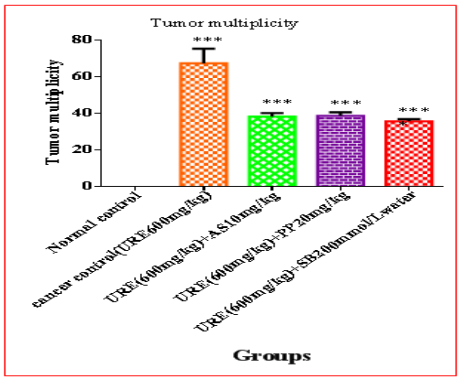

Figure 12: Tumor multiplicity variation observed in different group of BALB/c mice administered with atorvastatin, pantoprazole and sodium bicarbonate.

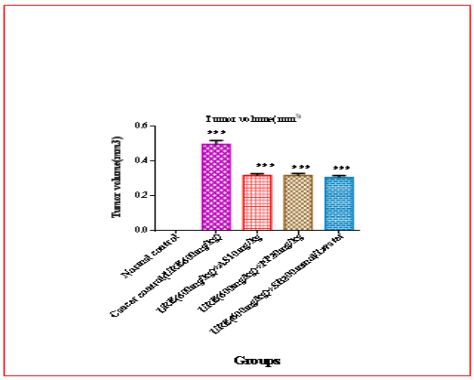

Figure 13: Tumor volume variation observed in different group of BALB/c mice administered with atorvastatin, pantoprazole and sodium bicarbonate.

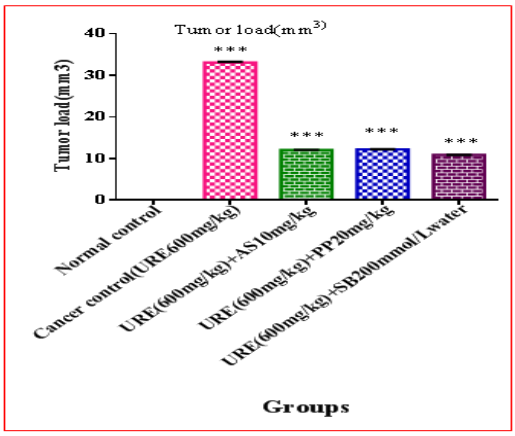

Figure 14: Tumor load variation observed in different group of BALB/c mice administered with atorvastatin, pantoprazole and sodium bicarbonate.

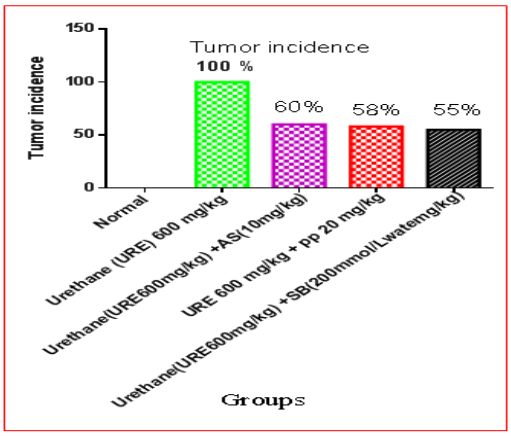

Figure 15: Tumor incidence variation observed in different group of BALB/c mice administered with atorvastatin, pantoprazole and sodium bicarbonate.

Table 5: Effect of Atorvastatin, Pantoprazole and Sodium bicarbonate on Relative lung weight in urethane induced lung carcinogenesis in BALB/c mice

Group	Treatment	Relative lung weight
Group I	0.5 % CMC 1ml/100 g oral	0.54666±0.04030
Group II	Urethane (URE) 600 mg/kg i.p.	0.87166±0.0614***
Group III	URE 600 mg/kg/ i.p.+ Atorvastatin 10mg/kg oral	0.06133±0.02076***
Group IV	URE 600 mg/kg i.p+ Pantoprazole 20mg/kg oral	0.5825±0.00847***
Group V	URE 600 mg/kg i.p+ Sodium bicarbonate 200mmol/L in drinking water	0.60666±0.0255***

Values are given in Mean±SEM for groups of six animals each.***P<0.001 denotes When cancer control was compared with the normal control and treated groups were compared with the cancer control.

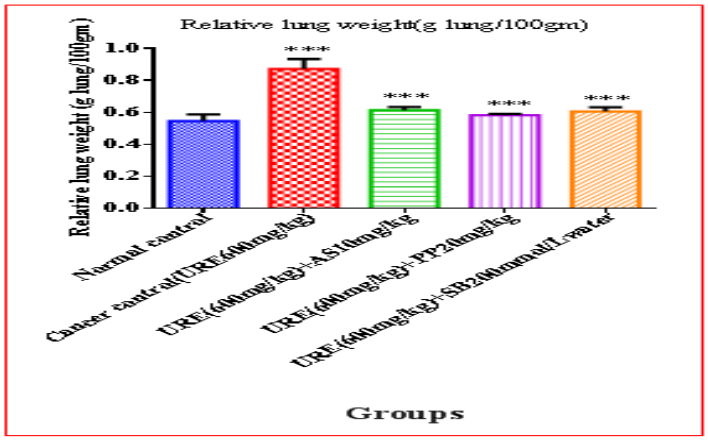

Figure 16: Relative lung weight variation observed in different group of BALB/c mice administered with atorvastatin, pantoprazole and

Effect of atorvastatin, pantoprazole and sodium bicarbonate on body weight of urethane induced lung carcinogenesis in BALB/c mice

The effect of *atorvastatin*, *pantoprazole* and *sodium bicarbonate* on body weight shown in Figure 1. It was observed that, there is no significant difference between these groups.

Effect of atorvastatin, pantoprazole and sodium bicarbonate on serum CEA& CRP of urethane induced lung carcinogenesis in BALB/c mice

The effect of *atorvastatin*, *pantoprazole* and *sodium bicarbonate* on serum CEA&CRP was shown in Table 1 and Figure 2-3. It was observed that administration of urethane (600mg/kg) to BALB/c mice increased the levels of serum CEA and CRP. The increased levels of serum CEA and CRP significantly decreased by treatment with *atorvastatin*, *pantoprazole* and *sodium bicarbonate* and were restored near to normal control.

Effect of atorvastatin, pantoprazole and sodium bicarbonate on blood and tissue parameters of urethane induced lung carcinogenesis in BALB/c mice

The effect of *atorvastatin*, *pantoprazole* and *sodium bicarbonate* on blood and tissue parameters was shown in Table 2, 3 and Figure 4-11. It was observed that, there was a significant increase in the blood and tissue parameters after treatment with urethane except SDH value. After treatment with *atorvastatin*, *pantoprazole* and *sodium bicarbonate* along with urethane significantly reduced the level of blood and tissue parameters. Table 2 and Figure 7 shows that urethane caused significant decreased in SDH and increase in LDH, lactate and pyruvate. Treatments with these drugs alter the levels, near to normal level.

Effect of atorvastatin, pantoprazole and sodium bicarbonate on tumor parameter of urethane induced lung carcinogenesis in BALB/c mice

The effect of *atorvastatin*, *pantoprazole* and *sodium bicarbonate* on tumor parameters was shown in Table 4 and Figure 12-15. It was observed that, these drugs try to restore the elevated tumor parameters such as tumor incidence, tumor multiplicity, tumor volume. This confirms that, this drug poses anti-cancer activity.

Effect of atorvastatin, pantoprazole and sodium bicarbonate on relative lung weight of urethane induced lung carcinogenesis in BALB/c mice: The effect of *atorvastatin*, *pantoprazole* and *sodium bicarbonate* on relative lung weight shown in Table 5 and Figure 16 It was observed that there was a significant increase in the weight of lung treated with urethane (600mg/kg). Generally, a urethane treated lung increase in weight due to tumor

formation. Treated with these drugs significantly reduced the relative lung weight and lung volume of BALB/c mice. Hence it poses significant anti-cancer activity.

Photographs of BALB/c mice lung: The histopathological studies observed in the microphotograph of lung for treated and control groups. Treated with atorvastatin, pantoprazole and sodium bicarbonate decreased the extent of neoplastic cells and interstitial inflammation caused by cancer causing agent urethane.

4. DISCUSSION

Cancer can be defined as an unregulated growth of cells arising from one cell. Killing of cancer cells without significant toxicity to normal cells is one of the most important considerations in cancer chemotherapy. Understanding the biological difference between cancer cells and normal cell is essential for design and development of anticancer drug with selective anticancer activity. Cancerous cell even in the presence of oxygen convert glucose to lactate by an altered energy metabolic pathway less efficient than oxidative phosphorylation. This along with increased degradation of glutamine leads to metabolic acidosis.

The end product of glycolysis, lactate is produced in large excess in tumors. The active secretion of lactic acid outside the tumor cell significantly contribute to the acidification of the extracellular mileu, in addition to other mechanism promoting tumor acidosis. This render the environment around the tumor tissue more suitable for colonization and invasion by cancer cells. Moreover, lactate also actively stimulates tumor cell migration by activation of β_1 integrin and angiogenesis following a stimulation of VEGF production in endothelial cells. Furthermore extracellular lactic acid was found to inhibit the ability of the immune system to eradicate aberrant cells, thus contributing to the immune escape phenomenon. Inhibition of lactic acid generation of cancer may be lead to inhibition of proliferation of cancerous cells by dual pathway via energy depletion, and by reduction of their immune-suppressive activity in the tumor microenvironment.

In present study, drug repositioning that take advantages of the existing data on pharmacokinetics was focused to identify a new target site. The three existing drug molecule were selected based on *in-vitro* studies for evaluation against urethane induced lung carcinogenesis. *Atorvastatin* is a HMG-CoA reductase inhibitor for the treatment of cholesterol and *pantoprazole* and *sodium bicarbonate* is used for the treatment of acidity.

Atorvastatin showed cytotoxic effect in different malignant cell in-vitro. Recently *atorvastatin* showed a potent mono-carboxylate transport inhibitory activity *in-vivo*. *Pantoprazole* and *sodium bicarbonate* based on pre-clinical evidence in cancer in *in-vitro* and *in-vivo* and recent studies on acidosis inhibition was selected for the study.

A strong link between lactate transport and lung carcinogenesis was recently found in urethane induced lung carcinogenesis, so urethane induced lung carcinogenesis model was selected for evaluation. In this present study, animals received urethane 600mg/kg ip once weekly for 10 weeks showed tumor incidence with increase in tumor load, tumor volume and tumor multiplicity. The relative lung weight was significantly increased and the histopathological report shows the presence of neoplastic cells. Markers of glycolytic stress (tissue lactate, pyruvate, LDH and serum lactate, pyruvate, LDH) were significantly increased. This might be due to proliferating tumor cells that actively uptake glucose through glycolytic pathway for their energy source that increase the formation of lactate and pyruvate in lung tissue cells and blood. The succinate dehydrogenase (SDH) is a mitochondrial enzyme, its level was found to be significantly decreased in urethane induced group. This might be due to compromised mitochondrial function in cancer cells.

Groups of animal treated with *atorvastatin*, *pantoprazole* and *sodium bicarbonate* showed a significant decrease in cancer incidence with decrease in tumor load, tumor volume and tumor multiplicity. The markers of glycolytic stress (tissue lactate, pyruvate, LDH and serum lactate, pyruvate, LDH) were significantly decreased and SDH levels were significantly increased compared to cancer control. This might be due to drug treated cells uptake glucose through glycolytic pathway comparatively lesser than the tumor cells for their energy source, that decrease the formation of lactate and pyruvate in blood and lung tissue. The histopathology of lung supported the biochemical parameters showed inflammatory infiltrates without any incidence of neoplastic cells. From the result, it was observed that *atorvastatin*, *pantoprazole* and *sodium bicarbonate* showed a significant protection against urethane induced lung carcinogenesis. This might be due to inhibition of mono-carboxylate transporters and metabolic acidosis by these drugs, thereby reduce the lactate production and acidosis in cancer cells.

5. CONCLUSION

The present study was to inhibit the lactate transport and acidosis by using *atorvastatin*, *pantoprazole* and *sodium bicarbonate* in urethane induced lung carcinogenesis model. These

drugs were procured from chemco and carried out the urethane induced lung carcinogenesis model in BALB/c mice.

The lung carcinogenesis was induced by using urethane 600mg/kg ip once weekly for 26 weeks. Then the treatment was carried out by using *atorvastatin* (10mg/kg), *pantoprazole* (20mg/kg), and *sodium bicarbonate* (200mmol/L in drinking water from the day of urethane administration once daily for 26 weeks. During the period of study the body weight of animals were recorded once weekly till the end of the study. At the end of the study the haematological (lactate, pyruvate, LDH, glucose) tissue (lactate, pyruvate, SDH) and morphological parameters are evaluated and histopathological examinations to evaluate the microscopical changes in the tissue.

From the haematological parameters show that treatment groups have significant decrease in lactate, pyruvate, glucose and LDH compared to cancer control group. The tissue parameter shows that treatment group have significant decrease in lactate, pyruvate and significant increase in SDH compared to cancer control group. The morphological parameter shows that, the treatment group have a significant decrease in cancer incidence decrease in tumour load, tumour volume and multiplicity.

The study demonstrated a significant protection by *atorvastatin*, *pantoprazole* and *sodium bicarbonate* against urethane induced lung carcinogenesis. These drugs may inhibit lactic acid generation and normalization of pH represents a very promising strategy for the development of new cancer therapies. Since these drugs may have potent anti-cancer activity in urethane induced lung carcinogenesis.

If the drug with well-known safety profile, pharmacokinetics and adverse events are repurposed for cancer treatment, this may provide affordable and safe therapeutic targets in oncology treatment.

This study serves as supporting evidence and can be scientifically exploited for future research in *in-vivo* cancer models focusing specifically on lactate transporter in cancer cells. So that these drugs can be repositioned as a single compound with chemotherapeutic agent for the treatment of patient with life threatening cancers. These findings provide new evidence for the mechanism by which this class of drugs may be acting on cancer cells.

6. ACKNOWLEDGEMENTS

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