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Fixed dose combination therapy loperamide and niacin ameliorates diethylnitrosamine-induced liver carcinogenesis in albino wistar rats

Ruqaiyah Khan[1], Imraan Kazmi,[2] Mohd. Afzal,[2] Fahad A. Al Abbasi,[3] Mushtaq Gohar,[3] Aftab Ahmad,[4] Vikas Kumar,[5*] Firoz Anwar[3*]

[1]Siddhartha Institute of Pharmacy, Dehra Dun, Uttarakhand, India

[2]Department of Pharmaceutical Sciences, Glocal University, Uttar Pradesh India

[3]Department of Biochemistry, Faculty of Science, King Abdulaziz University, Jeddah, Saudi Arabia

[4]Health Information Technology Department, Jeddah Community College, King Abdulaziz University, Saudi Arabia.

[5]Department of Pharmaceutical Sciences, Faculty of Health Sciences, Sam Higginbottom Institute of Agriculture, Technology & Sciences, Allahabad, Uttar Pradesh 211007, India

Abstract

Background

Hepatocellular Carcinoma (HCC) is among the most lethal cancers (five-year survival rates under 11%), which makes it the third most frequent cause of cancer related deaths in men and sixth in women. Still there are limited treatments available for majority of HCC at advanced stages. Systemic chemotherapy for advanced hepatocellular carcinomas, either as single-agent therapy or in combination, radiofrequency ablation or recently introduced tyrosine kinase inhibitors, e.g. sorafenib are some to bank upon.

Aim: This study is an attempt to evaluate the synergistic chemopreventive potential of Loperamide (5mg/kg) in combination with Niacin in hepatocarcinogenic rats. When challenged by a single diethylnitrosamine (DENA) (160 mg/kg).

Material and methods: The ability to treat hepatocellular carcinoma was measured by comparing biochemical serum markers such as glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), alkaline phosphatase (ALP), acid phophatase (AP), cholesterol (C), triglycerides (TG) and high density lipoproteins (HDL), total proteins (TPR), bilirubin and the specific marker for hepatocellular carcinoma such as alpha fetoprotein (AFP). The caspase-3 activity was also evaluated to decipher the potential of the drug as well as to explore the possible mechanism.

Results: The results have revealed the significant elevation of these parameters in DENA control group compared to normal control and the therapeutic groups. Casepase-3 activity was found to be highly elevated in the therapeutic group. Histopathology result also revealed severe the changes in hepatic tissues. Disease control animals showed central veins surrounded by extensive necrosis and inflammatory infiltrate, clusters of hepatocyte necrosis and the portal tract with bile duct proliferation and marked atypia. Liver section from loperamide with niacin control group shows the normal architecture of the liver, no necrosis was observed.

Conclusion

Our data indicates that this remarkable combination possess the potential for the treatment of hepatocellular carcinomas in rats exposed to DENA. Administration of Loperamide + Niacin relatively improved the biochemical parameters to values approximating those of the normal controls via increasing the Caspase-3 activity in them and inducing Apoptosis in cancerous cells.

Keywords: Hepatocellular carcinoma, DENA, Caspase-3 activity, Loperamide, Niacin.

Introduction

Liver cancer consists of several different primary hepatic malignancies, such as angiosarcoma, cholangiocarcinoma, hepatoblastoma and haemangiosarcoma, but hepatocellular carcinoma (HCC) is by far the most common type, accounting for 70%-85% of cases thus made it leading cause for cancer worldwide.[1] The only curative treatment is surgical resection or liver transplantation, but only10– 20% of patients are eligible for these procedures. The majority of hepatocellular carcinomas that present at an advanced stage are cancer which cannot be cured. Systemic chemotherapy available for advanced hepatocellular carcinomas is either as single-agent therapy or in combination, radiofrequency ablation or recently introduced tyrosine kinase inhibitors, e.g. sorafenib.[2] Despite all the treatment options when used as monotherapy, patients with HCC have a poor long term prognosis raising the economic burden. The overall per patient cost of HCC found to be $32,907 with an average annual HCC prevalence of 13,824 cases in United State alone, the total annual HCC burden thus estimated to be $454.9 million.[3]
Researchers have well established the potential hallmarks of cancer which include immortality, abnormal growth regulation, evasion of apoptosis, sustained angiogenesis, and invasion and metastasis.[4]

Research interest in opiate agonists, such as morphine, buprenorphine, dynorphin A, and Etorphine, explored the potential of producing cell apoptosis in immune and nervous systems through the activation of specific membrane-bound opioid receptors.[5-8] Loperamide is also a peripheral opiate agonist and has high affinity for the u subtype of the opioid receptor and one of the most used antidiarrheal drugs. Its effect on intestinal motility is well established.[9] The apoptosis-inducing activity of Loperamide has also been reported by the researchers.[10] Thus we presumed that loperamide might have potential capability to treat cancer via Apoptosis.[11]

The role of antioxidants in inhibiting the invasion and metastasis of liver cancer through control checking of Reactive Oxygen species (ROS) has been well established.[12,13] The relationship between supplemental vitamins and various types of cancer has been the focus of recent investigation, and supplemental vitamins have been reported to modulate cancer rates. A significant association has been demonstrated that progression of cancer and low levels of niacin.[14]

Niacin is a water-soluble vitamin and known as vitamin B3. It may also refer to either specifically to nicotinic acid or to the total amount of nicotinic acid and nicotinamide in the diet.

Niacin is precursor for synthesis of nicotinamide adenine di nucleotide NAD^+ synthesis and NAD^+ has shown to be free radical scavenger possesing antioxidant properties.[15,16] Nubohiro and his colleagues have well proved the anti invasive potential of Niacin demonstrating the inhibition of AH109A cells by suppressing the ROS potentiated invasive capacity of the Hepatoma cells.[17] Niacin is a precursor for NAD^+, ATP and endogenous inhibitor of PARP-1 switchs the mode of cell death from necrosis to apoptosis via caspases 3 dependent pathway.[18]

For the potential and synergistic effects, combination therapies pave as alternative treatment for HCC. In this research we have investigated the potential synergestic chemopreventive effect of the combination of Loperamide and Niacin in DENA induced wistar rats thus moving in the next level in the direction of exploring the chemopreventive potential from invitro to invivo. This novel combination may prove to be effective novel approach in the field of chemotherapy for the treatment of hepatocarcinogenesis.

Material and Methods

Drugs and chemicals

Loperamide and Niacin was provided as a gift sample from Siddhartha Institute of Pharmacy, Dehradun; DENA was procured from Sigma–Aldrich Chemicals Co., St. Louis, USA and Chloroform and Diethyl ether from S.D. Fine Chem. Ltd., Mumbai and all the chemicals were of analytical grade.

Animals

Adult, healthy, male Wistar albino rats weighing 100–125 g were procured in polypropylene cages in the animal house facility of Siddhartha Institute of Pharmacy for the present protocol under controlled conditions of temperature (22+ 3^0C) and light (14:10 h light and dark cycle) and provided with balanced pallet diet. The protocol was approved by the Institutional Animal Ethics Committee (IAEC) as per the guidance of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA); Ministry of Social Justice and Empowerment, Government of India

Experimental Design

The rats (Wistar albino) were acclimatized and randomly divided into eight groups each having 6 rats for a 12 week study. Group-I rats served as normal control and were treated with saline orally. Group-II rats were administered a single dose of DENA, Group-III rats served as Loperamide control, Group-IV as Niacin control, Group-V as Loperamide and Niacin control,

After 7th day of DENA (160 mg/ kg) administration HCC was induced, Group-VI served as DENA and Loperamide control, Group-VII as DENA and Niacin control, Group-VIII served as a therapeutic group DENA + Loperamide + Niacin. The dose of Loperamide was selected as per the used dose in various researches in liver cancers and the treatment was started as soon as liver cancer was developed.

Estimation of biochemical parameters

Blood samples were collected on the termination day of the experiment from the retro-orbital plexus under light ether anesthesia without any anticoagulant and were allowed to stand for 30 min at room temperature, centrifuged at 2500 rpm for 10 min to separate the serum. Estimation of serum SGOT, SGPT, ALP, TC, TG, HDLand BIL was performed using standard kits (Nicholas India Pvt. Ltd.) with semi-auto analyzer (photometer 5010, Nicholas Pvt. Ltd.). Serum alpha-feto protein (AFP) was estimated by the method described by Premalatha and Sachdanandam.[19]

Caspase-3 activity in myocardium

The activity of caspase-3 was determined by the detection of chromophore *p*-nitroanilide after cleavage from the labeled substrate DEVD- *p*-nitroanilide. In brief, 50µL supernatant from homogenized tissue with cooled lysis buffer was used from each sample and 50 µL of Reaction Buffer was added to each sample. Then, 5µL of the 4mM DEVD-pNA substrate (200 50µM) was added and incubated at 37°C for 30 min to permit a dissociation of p- nitroanilide) (pNA) from the conjugate DEVD-pNA. The activity was read by Elisa at 405 nm using 96 well plate.[20]

Estimation of Survival

Survival rate of animals for all groups were determined by observing their mortality rate during the period of experiments.[21]

Histopathological examination

Small pieces of liver tissues were collected in 10% formalin for proper fixation. These tissues were processed and embedded in paraffin wax. Section of 5- 6 microns in thickness were cut and attained with hematoxylin and eosin. All the sections of the tissues were examined under microscope for the analyzing the altered architecture the liver tissue due to DENA challenge and improved liver architecture due to with test drug Loperamide, Niacin and Loperamide and Niacin together. These were examined under the microscope for histopathological changes such as congestion, hemorrhage, necrosis, inflammation, Infiltration, kuffer cell and sinusoids and

photographs were taken. The photographic figures are given as an evidence for the improved architecture of the liver due to pretreatment with test drug and the models of our study (Conducted at Chabra Pathology Lab, Dehradun).

Statistical analysis

Statistical analysis was carried out using Graph pad prism 5.0 (Graph pad software, San Diego, CA, USA). The results were expressed as Mean ± S.E.M. Statistical significance between more than two groups was tested using one-way ANOVA followed by Tukey's multiple comparison tests. Values of $p < 0.05$ were regarded as significant.

Results

Animal weight

Disease control group showed significant reduction in body weight as compared to a normal control group. In Therapeutic groups the body weight was significantly increased as compared to Disease control group (Table 1).

Liver profile study

Serum glutamate pyruvate transaminase (SGPT/ALT)

In DENA control group the SGPT levels were elevated significantly ($p<0.001$) as compare to normal control group. DENA +Loperamide control group reduced the elevated SGPT significantly ($p<0.01$) when compared to DENA+ Niacin control which found to be slightly significant ($p<0.05$). Treatment with Loperamide in combination with Niacin decreased significantly ($p<0.001$) the elevated levels as compared to DENA control group (Table 1).

Serum glutamate oxaloacetate transaminase (SGOT/AST)

In DENA control group the SGOT levels were found to be increased significantly ($p<0.001$) as compare to Normal control animals. DENA control group when compared to DENA +Loperamide control group reduced the elevated SGOT significantly ($p<0.01$) and when compared to DENA+Niacin control it was slightly significant ($p<0.05$).Treatment with Loperamide in combination with Niacin decreased significantly ($p<0.001$) the elevated levels as compared to DENA control group (Table 1).

Alkaline Phosphatase (ALP)

ALP levels were significantly ($p<0.001$) increased in DENA control as compare to Normal control group. DENA control group when compared to DENA +Loperamide control group reduced the elevated ALP significantly ($p<0.01$) and when compared to DENA+Niacin control,

the result obtained was not significant. Treatment with Loperamide in combination with Niacin decreased significantly ($p<0.005$) the elevated levels as compared to DENA control group (Table 1).

Total Cholesterol (TC)

The all results were not significant (Table 1).

Triglycerides (TG)

In DENA control group the TG levels were elevated significantly ($p<0.001$) as compare to Normal control group, DENA control group when compared to DENA +Loperamide control group and DENA+Niacin control group shows no significant results. Treatment with Loperamide in combination with Niacin also had no significant effect as compared to DENA control group (Table 2).

High Density Lipoprotein (HDL)

In DENA control group the HDL levels were elevated significantly ($p<0.001$) as compare to Normal control group, DENA control group when compared to DENA +Loperamide control group reduced the elevated HDL significantly($p<0.01$) and when compared to DENA+ Niacin control it was not significant. Treatment with Loperamide in combination with Niacin decreased significantly ($p<0.001$) the elevated levels as compared to DENA control group (Table 2).

Bilirubin (BIL)

In DENA control group the BIL levels were elevated significantly ($p<0.001$) as compare to Normal control group, DENA control group when compared to DENA +Loperamide control group reduced the elevated BIL significantly($p<0.001$) and when compared to DENA+ Niacin control it was not significant. Treatment with Loperamide in combination with Niacin decreased significantly ($p<0.001$) the elevated levels as compared to DENA control group (Table 2).

Alfa Feto Protein (AFP)

In DENA control group the AFP levels were elevated significantly ($p<0.001$) as compare to Normal control group, DENA control group when compared to DENA +Loperamide control group reduced the elevated HDL significantly ($p<0.001$) and when compared to DENA+Niacin control it was slightly significant ($p<0.05$). Treatment with Loperamide in combination with Niacin decreased significantly ($p<0.001$) the elevated levels as compared to DENA control group (Table 2).

Caspase-3 activity

Our findings clearly demonstrate that therapeutic group Loperamide+ Niacin dramatically increased ($p<0.001$) the caspase-3 activity by 5 folds when compared to the Normal control and diseased control group. Loperamide control group and Niacin control group also showed significant ($p<0.01$) rise in caspase activity (figure 1)

Survival

A significant survival increase was observed for rats bearing Loperamide and Niacin combination as compared to Loperamide and Niacin alone in DENA induced HCC It is important to emphasize that all the surviving animals presented complete tumor regression. These results explain the great effectiveness of Loperamide and Niacin as an antineoplastic agent, confirmed through the data obtained in the experimental rats (figure 2).

Histopathological study

Liver sections of the normal control group showed normal liver histology with unremarkable central veins, no evidence of hepatocyte injury or fibrosis or dysplasia or malignancy noticed. Disease control animals showed central veins surrounded by extensive necrosis and inflammatory infiltrate, clusters of hepatocyte necrosis and the portal tract with bile duct proliferation and marked atypia. The tumor cells resembling hepatocytes show pleomorphism and were seen 2-8 cell, wide trabeculae which are separated by endothelium lined sinusoidal spaces. The prophylactic group showed periportal inflammation with conspicuously dilated blood vessels and ballooning degeneration mononuclear infiltrates associated with regenerative cellular changes of the adjacent hepatocytes, mild bile duct proliferation and intra-acinar inflammatory cell infiltrates was observed. Liver section from loperamide with niacin control group shows the normal architecture of the liver, no necrosis was observed (figure 3).

Discussion

Hepatocellular carcinoma (HCC) accounting for 70%-85% of cases of liver cancer and is the fifth most common cause of cancer and the third leading cause of cancer-related deaths worldwide.[22] Present study has been initiated to investigate loperamide and Niacin in combination play an important role as a potent anti-cancer activity in hepatocellular carcinomas induced by diethylinitrosamine (DENA), a potent initiator and hepatocarcinogen, in rats.

DENA induced Hepatocellular damage clearly demonstrated that DENA significantly ($p<0.001$) elevated the levels of liver enzymes i.e. SGPT, SGOT, and bilirubin and caused severe

histopathological lesions in liver tissues. The elevated level of the liver enzymes may be due to leakage from damaged tissues, overproduction and leakage in blood, decreased hepatic clearance leading to viral hepatitis, granulomatous hepatitis, infiltration syndrome, hyper-bilirubinemias, physiological jaundice and nonalcoholic fatty liver disease (NAFLD).[23,24] It has also been observed and established by the researchers that SGPT, SGOT, serum bilirubin level elevates significantly after DENA exposure in the experimental animals.[25] In present study serum SGPT and SGOT levels elevated significantly ($p<0.001$) in all groups exposed to DENA as compared to the NC group. While the Therapeutic group loperamide with niacin SGOT SGPT and Serum bilirubin levels brought towards the normal levels. These results firmly established the role of loperamide with niacin as a chemopreventive agent in DENA induced Hepatocellular carcinoma.

Triglyceride levels elevated significantly ($p<0.001$) in all groups exposed to DENA as compared to the NC group. Interestingly, treatment with Loperamide (5mg/kg) and Niacin (*ad libitium*) combination significantly reduced ($p<0.001$), ($p<.001$) respectively triglyceride level. These results are firm indications that Loperamide and niacin combination maintains the lipid profile in DENA induced liver cancer and inhibit the cell proliferation.

Previously the researchers has established that Plasma lipid metabolism are associated with hepatocellular carcinomas alterations in lipid metabolism,[26] affects cellular function and growth, further development of hepatocyte nodules in rat liver has been found with changes in lipid parameters and oxidative status.[27] Alteration in plasma lipid profile in malignant tissue are of important due to the effect on membrane integrity, fluidity and regulation of cellular process related to growth and cell survival.[28,29]

The present research concluded that therapeutic group (Loperamide + Niacin) maintained the lipid profile, hence it can be suggested that they may play the role in inhibition of carcinoma progression.

Apoptosis is a programmed cell death which is a complex biological process enabling the removal of unwanted cells during development, normal homeostasis or disease.[30] Problem with the regulation of apoptosis have been correlated with a number of diseases and is also well established reason for the occurrence of cancer. Due to mutation cancerous cells escape from normal cellular signals which use to regulate their growth. Thus, cancer cells are more proliferative than normal.[31] The process of apoptosis paves the interest and understanding for the development of treatments for this disease. This research paper is first to report the synergistic

apoptotic activity of Loperamide +Niacin in different therapeutic animal model. It is clearly evident through our results that demonstrated that therapeutic group Loperamide+ Niacin dramatically increased ($p<0.001$) the caspase-3 activity by 5 folds when compared to the Normal control and diseased control group that our drug combination significantly increases apoptosis which is revealed by increase in caspase-3 activity. Moreover, AFP is a serum protein, shows higher specificity for HCC.[32] AFP has to be considered as 'the gold standard' for HCC serum markers.

In present experimental protocol it has been observed that serum AFP level were increased in the disease control group as compared to normal controls, treatment with loperamide 5mg/kg + niacin significantly reduced ($p<0.001$) the serum AFP levels as compared to disease controls Moreover Loperamide works as a anti-cancer agents due to their anti-angiogenic and apoptotic properties. And niacin work as anti-cancer agents due to their anti-angiogenic, anti oxidant, anti-inflammatory, anti diabetic and calcium releasing properties (figure 4).[33]

The survival graph further provided evidences towards the potent effect of Loperamide and Niacin as combination. From the outcomes of the present research done on the experimental animals it is concluded that the combination may be proved a boon for the treatment of hepatocellular carcinoma. But further exploration of the combination needs to be done i.e the clinical studies.

Conclusion

Data from the study suggests that Loperamide and niacin together can posses synergestic chemopreventive action. Combination of Loperamide (5 mg/kg) and niacin suppress the tumor lesions and decrease the biochemical markers which were elevated in HCCs.

The clinical application of loperamide and niacin combination would benefit the cancer patients due to decrease their therapeutic cost significantly. In conclusion loperamide and niacin combination were found to be a potential anti-tumor agents with apoptosis inducing activity, anti- angiogenesis, anti-proliferation activity and free radicals scavenger.

This finding provides new insight into the existing drugs and may help to facilitate the development of anti-tumor agents.

Competing interests

The authors declare that they have no competing interests.

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Table 1: Effect of Loperamide in combination with Niacin on serum SGOT SGPT, ALP and AFP level of animals

S. no.	Groups	Body weight	SGOT (mg/dl)	SGPT (mg/dl)	ALP (mg/dl)	AFP (mg/dl)
1	Normal control	130.5±3.2	87.50 ±8.7	112.2±8.76	135.0±2.23	0.28±0.0254
2	DENA control	97.5±5.4$^{###}$	273.7±2.04$^{###}$	230.0±7.10$^{###}$	254.0±5.89$^{###}$	1.598±0.153$^{###}$
3	Loperamide (L) control	117.6±5.2***	98.7±1.22***	135.3±5.51***	149.3±3.36***	0.35±0.03***
4	Niacin control (Nia)	120.7±4.0***	102 ± 3.22***	112.5±5.43***	121.5±1.58***	0.45±0.02***
5	(L+ Nia) control	105.5±6.0***	95.40±8.94***	150.8±7.97***	195.5±2.02***	0.46±0.02***
6	Dena+(L) control	110±3.4***	155.7±1.48***	189.5±8.22**	182.2±6.59***	0.95±0.04***
7	Dena+ (Nia)control	91.2±6.7**	201.5±5.55**	200.4±6.31*	230.5±1.02^{ns}	0.76±0.03*
8	Dena+ (L) + (Nia) control	120.7±6.2***	130.0±7.35***	158.1±7.35***	190.0±9.95***	0.382±0.03***

Data showing comparison of serum SGOT, SGPT, ALP and TC level of animals in normal control (NC), disease control (DC), and treated group. Values are expressed in mean ± SEM. n=6 (#) Groups compared to normal control; (*) Groups compared to DENA control.*ns –not significant;* (P<0.05); **(P<0.01); ***(P<0.001)*.

Table 2: Effect of Loperamide in combination with Niacin on serum TG, HDL, BIL and TC level of animals

S. no.	Groups	TG (mg/dl)	HDL (mg/dl)	BIL (mg/dl)	TC (mg/dl)
1	Normal control	75.54 ±3.83	45.67±2.15	0.69±0.363	96.1±1.90
2	DENA control	141.01±2.74$^{###}$	18.00±3.5$^{###}$	1.00±0.59$^{###}$	141.3±1.50**
3	Loperamide (L) control	105.83 ± 8.9**	43.5±2.5***	0.46±0.03***	102.2±3.5^{ns}
4	Niacin control (Nia)	99.5 ± 5.2*	43.8±2.1***	0.34±0.04***	100.5±3.5^{ns}
5	(L+ Nia) control	120.8±7.54*	31.67±2.6*	0.45±0.03***	115.5±2.5^{ns}
6	Dena+(L) control	100.0±6.13 *	35.0±1.9**	0.75±0.04***	130.0±5.0^{ns}
7	Dena+ (Nia)control	99.0±5.0 *	30.0±1.8^{ns}	0.99±0.11^{ns}	131±4.42^{ns}
8	Dena+ (L)+(Nia) control	84.7±2.5***	42.5±1.62***	0.70±0.08***	115.3±2.4***

Data showing comparison of serum TG, HDL, TB and AFP level of animals in normal control (NC), disease control (DC), and treated group. Values are expressed in mean ± SEM. n=6 (#) Groups compared to normal control; (*) Groups compared to DENA control.*ns –not significant;* (P<0.05); **(P<0.01); ***(P<0.001)*.

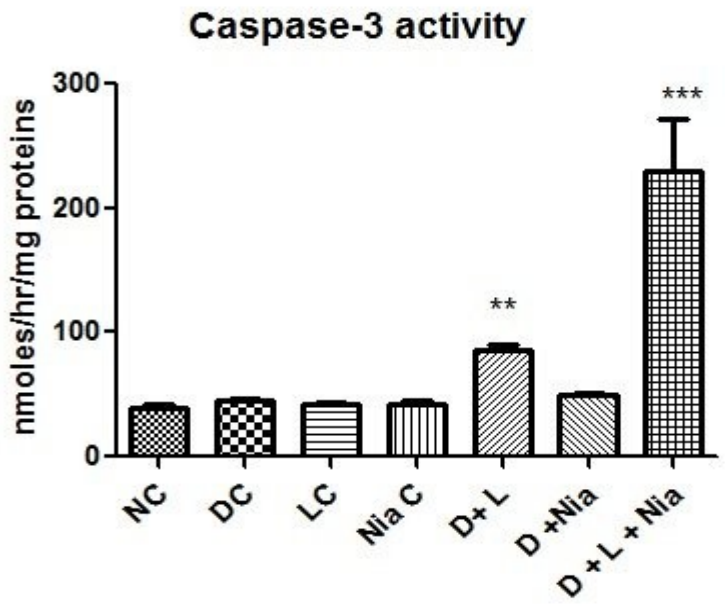

Figure 1: Caspase-3 activity in HCC. Values are expressed s mean + SEM of 6 animals: ** significant compared with DC alone group, P< 0.01. NC: Normal Control; DC: Dena control; LC: Loperamide Control; Nia C: Niacin Control; D+ L +Nia : Dena + Loperamide + Niacin control.

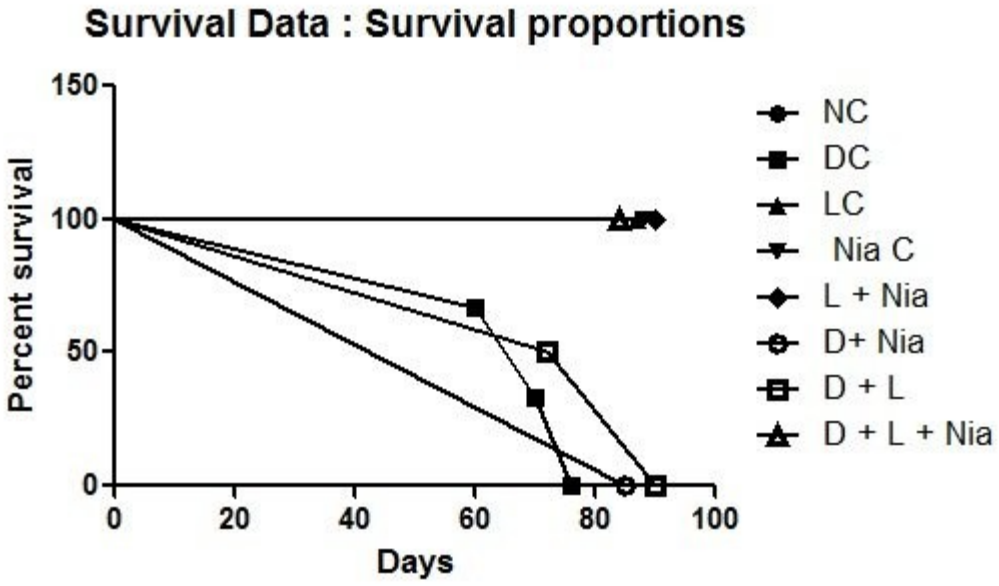

Figure 2: Survival graph for various control groups

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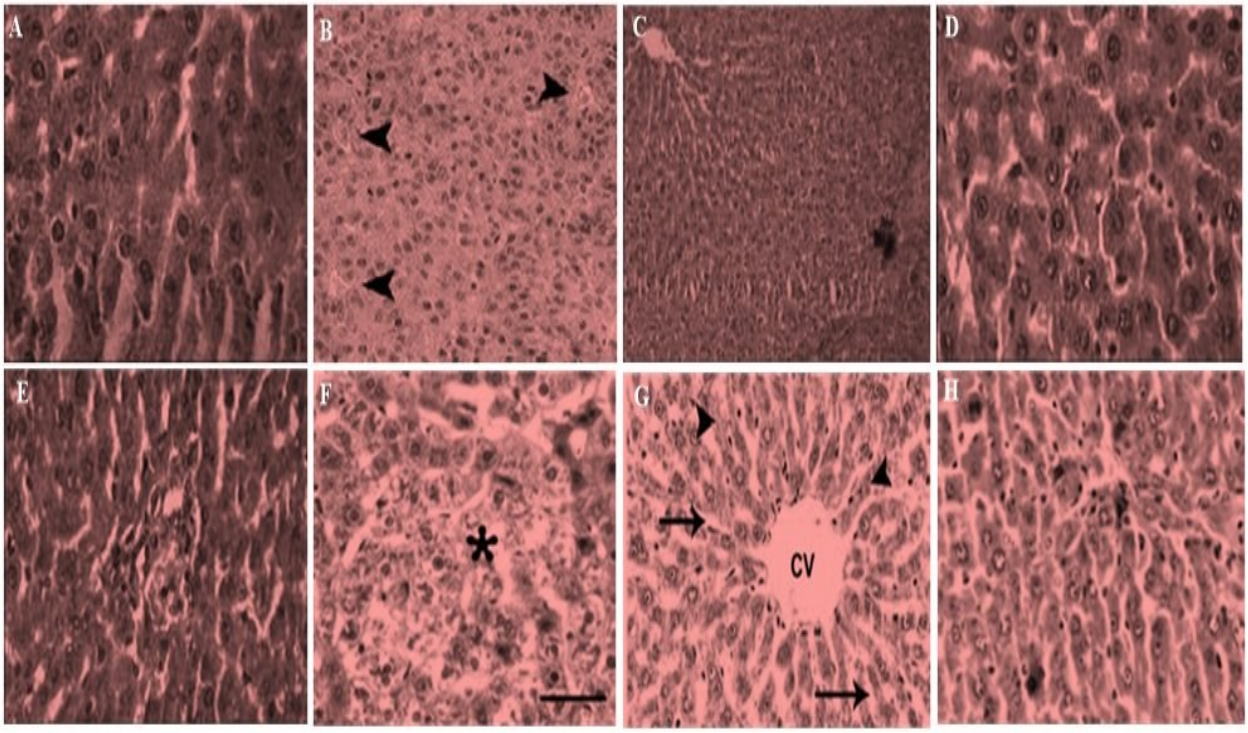

Figure 3: Histological images (40X) show the various groups of protocol: (A) liver from normal untreated control (group 1).showing normal hepatic cords. (B) cancerous liver from rat in DENA control (group 2), showing pseudoacini (concave arrowheads); (C) hepatic tissues from rat treated with Loparamide only (group 3) displaying moderate to severe sinusoidal (arrows) and venous (concave arrowhead) congestion; (D) hepatic tissues from rat injected with Niacin only showing normal hepatic parenchyma (group 4); (E) hepatic tissue of rats injected with loparamide and niacin in combination showing the normal lobular organization represented by central vein (CV), hepatic cords (concave arrowheads) and sinusoids (arrows) (group 5); (F) hepatic tissues from rat injected with DENA and treated with loparamide (group 6) showing cancerous focus (asterisk) along with ballooning degeneration (group 6); (G) liver from rats treated with Niacin only after DENA administration (group 7) reparative changes along with normal lobular organization represented by central vein (CV), hepatic cords (concave arrowheads) and sinusoids (arrows); (H) hepatic tissues from rats injected with DENA and treated with loparamide and niacin in combination (group 8) showing significant reparative changes along with improved hepatic cords.

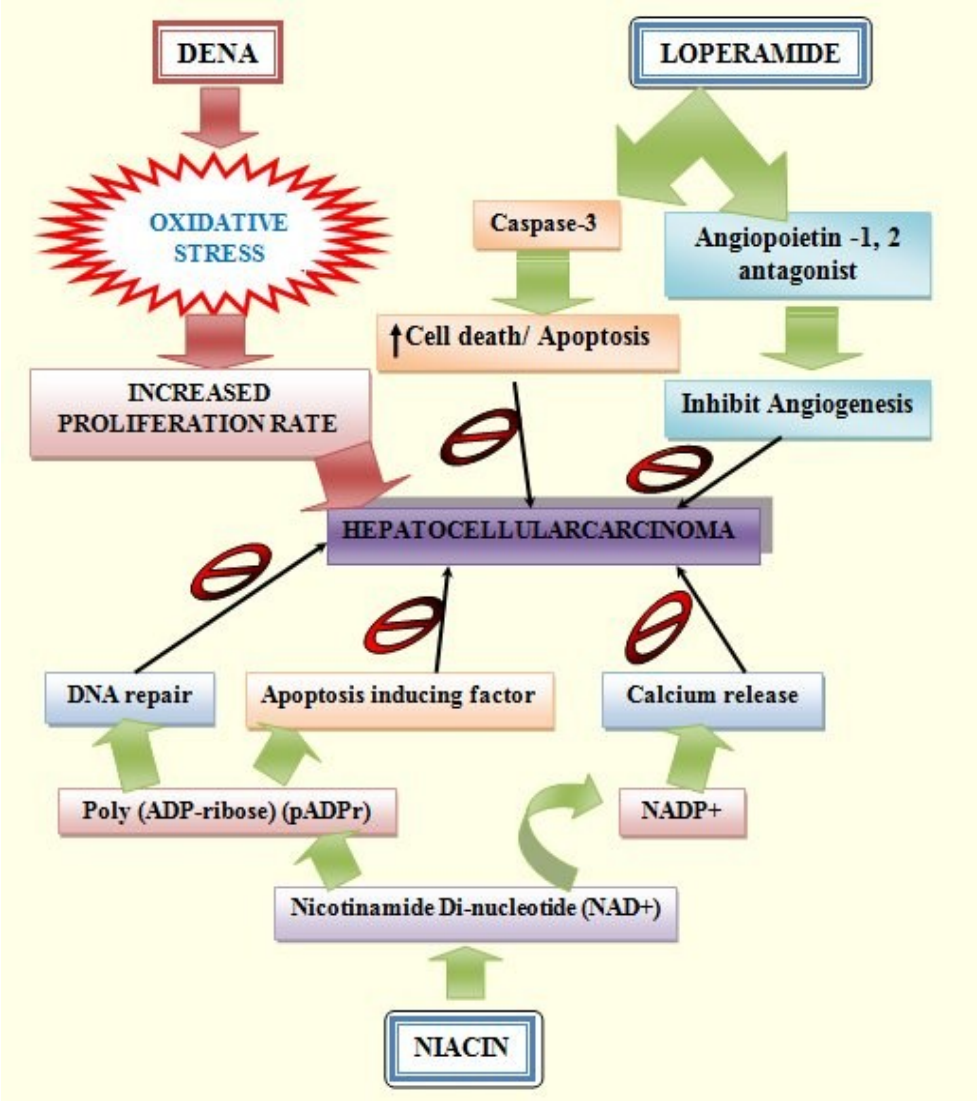

Figure 4: Mechanism illustration of Loperamide and Niacin