ENHANCED ANTICANCER POTENTIAL OF IMIPRAMINE IN CONJUGATION WITH NIACIN IN DENA INDUCED HEPATOCELLULAR CARCINOMA

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ABSTRACT

Hepatocellular carcinoma (HCC) is constantly seeking the interest of researchers as an untreatable and fatal disease, attaining second position so far worldwide. Our study provides us an opportunity to explore new potential therapeutic combinations in this regard. We hypothesized that the anticancer activity of imipramine, which is used as an antidepressant drug, can be potentiated with an adjuvant, niacin, thereby providing a better treatment approach. The combination turned out to show beneficial alterations in various liver function test, caspase 3 (antiapoptotic) activity, tumor growth and survival profile of animals, precisely in our therapeutic group involving imipramine and niacin combination post diethyl nitrosamine (DENA) dose and hepatocellular carcinoma induction. Results demonstrated a remarkable restoration in all liver enzymes in the therapeutic groups.

Keywords: DENA, imipramine, niacin, liver enzymes, caspase 3 activity

INTRODUCTION

Liver cancer in men is the fifth most frequently diagnosed cancer worldwide and the second most frequent cause of cancer death[1,2]. In women, it is the seventh most commonly diagnosed cancer and the sixth leading cause of cancer death[2]. The distribution of (HCC) hepatocellular carcinoma is heterogeneous with a high prevalence seen in Asia[3] and eighty percent of the burden is borne by countries in Asia and sub-Saharan Africa[4]. The highest liver cancer rate in the world is in China, according to the cancer registry reporting[5]. Due to potential side effects and progressively increasing economic burden of currently available drugs and due to the strong resistance against standard chemotherapeutic drugs in HCC treatment showed by many hepatoma cells[6], there is a need to develop new anticancer agent[7].

Imipramine is an important antidepressant agent frequently prescribed in psychiatric disorders. It contains a characteristic three-ring nucleus and is a member of the tricyclic antidepressants group. Imipramine acts as an inhibitor of serotonin and norepinephrine reuptake[8, 9]. Imipramine had shown anticancer activity in various research models, especially cancer cell lines[10]. Imipramine resulted in the inhibition of PI3K/Akt/mTOR signaling, reduction of clonogenicity and induction of cell death when exposed to U-87MG cells in a study[11]. Imipramine in dose dependent manner (50μM) induces apoptosis in human peripheral lymphocytes; cytotoxic T-lymphocytes were more prone to undergo apoptosis than were T-helper cells[11]. Activation of caspase induced by imipramine was preceded by the hyper generation of intracellular reactive oxygen species (ROS). These results suggested that imipramine may induce apoptosis via a caspase-3-dependent pathway[12]. Niacin is a water-soluble vitamin and known as vitamin B3. The term niacin may refer either specifically to nicotinic acid or to the total amount of nicotinic acid and nicotinamide in the diet. Niacin is a precursor for synthesis of nicotinamide adenine di nucleotide NAD^+ synthesis and NAD^+ has shown to be a free radical scavenger possessing antioxidant properties[13,14]. Niacin as a precursor for NAD^+, ATP and endogenous inhibitor of PARP-1 switches the mode of cell death from necrosis to apoptosis via caspase 3 dependent pathway[15].

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MATERIALS AND METHODS

Drugs and chemicals

Imipramine and niacin were provided as gift samples from Dr. Firoz Anwar, Principal & Dean (Research & Academics) Siddhartha Institute of Pharmacy. DENA (diethyl nitrosamine) was purchased from Sigma-Aldrich Chemicals Co., Singapore, chloroform and diethyl ether from S.D. Fine Chem. Ltd. Mumbai. All the chemicals were of analytical grade.

Animals

The study was carried out in the Department of Pharmacology, Siddhartha Institute of Pharmacy. Adult, healthy, male Wistar albino rats weighing 100–125 g were procured from the central animal house facility. The rats were housed in groups in polypropylene cages under controlled conditions of temperature (22+ 3°C) and light (14:10 h light and dark cycle) and provided with balanced pallet diet and water. The protocol was approved by the Institutional Animal Ethics Committee (IAEC) SIP/ADM/3247/2018 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.

Induction of hepatocarcinoma

Liver cancer was induced by a carcinogenic dose of 200 mg kg^{-1} body weight, I.P. DENA when associated with fasting/refeeding[16].

Experimental design

The rats were acclimatized and randomly divided into eight groups each having 5 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 imipramine control, Group-IV as niacin control, Group-V as imipramine and niacin control, After 7th day of DENA (200 mg kg^{-1}) administered and determination of it by checking alpha feto proteins levels confirming induction of HCC, Group-VI rates were made to serve as DENA and imipramine control, Group-VII as DENA and niacin control, Group-VIII served as a therapeutic group DENA + imipramine + niacin.

Estimation of biochemical parameters

Blood samples were collected on termination day of the experiment from retro-orbital plexus under light ether anesthesia without any anticoagulant and allowed to stand for 30 minutes at room temperature, then centrifuged at 2500 rpm for 10 minutes to separate the serum. The serum obtained was kept at 2° - 4°C for further use. Estimation of serum SGOT, SGPT, ALP, TC, TG, HDL, and BIL were performed using standard kits (Nicholas India Pvt. Ltd.) with semi-auto analyzer (photometer 5010, Nicholas India Pvt. Ltd.).

Statistical analysis

Results were expressed as mean ± S.E.M. Statistical significance between more than two groups was tested using one-way ANOVA followed by the Bonferroni multiple comparisons test or unpaired two-tailed student's t-test as appropriate using computer-based fitting program (Prism, Graphpad). Differences were considered to be statistically significant when $p < 0.0005$. Survival curves were represented by the Kaplan–Meier method and the statistical comparisons among the groups were carried out using the Mantel–Haenszel Log-rank test for non-parametric procedures.

RESULTS

Liver profile study

Serum glutamate pyruvate transaminase (SGPT)

SGPT levels were found to be significantly increased ($p<0.001$) in DENA control animals when compared to normal control animals, DENA + imipramine administered animals reduced the elevated SGPT significantly ($p<0.01$) when compared to DENA control; same results were observed in DENA+ niacin control ($p<0.05$). Treatment with imipramine in combination with niacin reduced the elevated SGPT significantly ($p<0.001$) as compared to DENA control group (Table I).

Serum glutamate oxaloacetate transaminase (SGOT)

Significant elevation was noted in SGOT levels of DENA control ($p<0.001$) as compared to normal control group, on the other hand, DENA + imipramine control group reduced the elevated SGOT significantly ($p<0.01$) in comparison to DENA control and same was the case with DENA+ niacin control but slightly significant ($p<0.05$). Imipramine and niacin combination in turn produced significantly ($p<0.001$) reduced levels as compared to DENA control group (Table I).

Alkaline phosphatase (ALP)

DENA control group showed elevated ALP levels ($p<0.001$) as compared to normal control group; DENA

+ imipramine control group reduced the elevated ALP significantly ($p<0.01$) in comparison to DENA control; other therapeutic group of DENA+ niacin was unable to show any alteration. Treatment of HCC with imipramine and niacin combination significantly ($p<0.005$) decreased the elevated levels as compared to DENA control group (Table I).

Total cholesterol (TC)

All results were not significant (Table I).

Triglycerides (TG)

Significant alteration was noted in TG levels which were found to be elevated significantly ($p<0.001$) in DENA control as compared to normal control group, though compared to DENA + imipramine control group and DENA+ niacin control group showed no significant alterations. Treatment with imipramine in combination with niacin also was unable to prove its effect on TGs level when compared to DENA control group (Table II).

High density lipoprotein (HDL)

Elevated HDL levels were observed significant to $p<0.001$ in DENA control as compared to normal control group, DENA + imipramine control group when compared to DENA control reduced the elevated HDL significantly

Table I: Effect of imipramine in combination with niacin on serum SGOT, SGPT, ALP and TC levels of animals

S. no.	Group	SGOT (mg dL^{-1})	SGPT (mg dL^{-1})	ALP (mg dL^{-1})	TC (mg dL^{-1})
1	Normal control	136.8 ±7.19	133±4.17	158±6.67	97.72±2.44
2	DENA control	270.4±5.98$^{###}$	311.8±15.19$^{###}$	306.4±7.40$^{###}$	114.0±2.28$^{###}$
3	Imipramine control (IMP)	134±9.92***	262.2±4.2^{ns}	175.6±5.53***	108.2±3.2^{ns}
4	Niacin (nia) control	155± 6.20***	145.0±12.91***	156.6±9.08***	105.5±3.5^{ns}
5	IMP+nia control	224±7.2***	237.4±14.19*	157.0±6.17***	96±2.34*
6	Dena+IMP control	250±4.07^{ns}	269±17.01^{ns}	224.0±19.21***	123.2±3.73^{ns}
7	Dena+nia control	303±6.0*	285±16.87^{ns}	276.8±4.24^{ns}	144.8±7.1 ***
8	Dena+IMP+nia control	230±5.8**	250±14.08^{ns}	231.2±4.28***	110.5±3.4^{ns}

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=5 (#) Groups compared to normal control; (*) Groups compared to DENA control.ns –not significant; * (P<0.05); **(P<0.01); ***(P<0.001)*

Table II: Effect of Imipramine in combination with niacin on serum TG, HDL, AFP and caspase 3 level of animals

S. no.	Group	TG (mg dL^{-1})	HDL (mg dL^{-1})	AFP (mg dL^{-1})	Caspase-3(nm mg^{-1})
1	Normal control	63.02 ±3.43	66.40±3.8	0.18±0.03	55±4.5
2	DENA control	136.3±3.72$^{###}$	34.13±2.5	0.52±0.07$^{###}$	47±2.4$^{###}$
3	Imipramine control (IMP)	124.8 ± 2.7^{ns}	53.60±2.9***	0.22±0.03***	51±1.4**
4	Niacin (nia) control	69.4 ± 3.72***	61.00±5.07***	0.10±0.03***	49±3.8**
5	IMP+nia control	128.0±3.42^{ns}	60.80±2.26***	0.10±0.03***	49±2.0*
6	Dena+IMP control	125.6±5.06^{ns}	37.71±2.10*	0.38±0.03^{ns}	60±3.4*
7	Dena+nia control	93.0±2.8***	36.20±2.28*	0.46±0.11^{ns}	63±4.5**
8	Dena+IMP+nia control	105.8±2.3***	44.44±2.82*	0.30±0.07*	74±2.7***

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=5(#) Groups compared to normal control; (*) Groups compared to DENA control.ns –not significant; * (P<0.05); **(P<0.01); ***(P<0.001)*

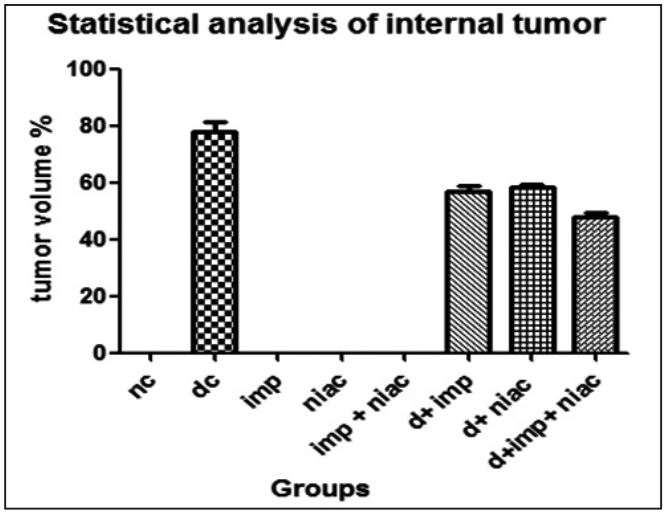

Fig. 1: Tumor growth after 7th day of cancer induction and its effective reduction in tumor volume till the terminating day of protocol by imipramine alone, niacin alone, imipramine and niacin in combination (given as tumor volume in percent)

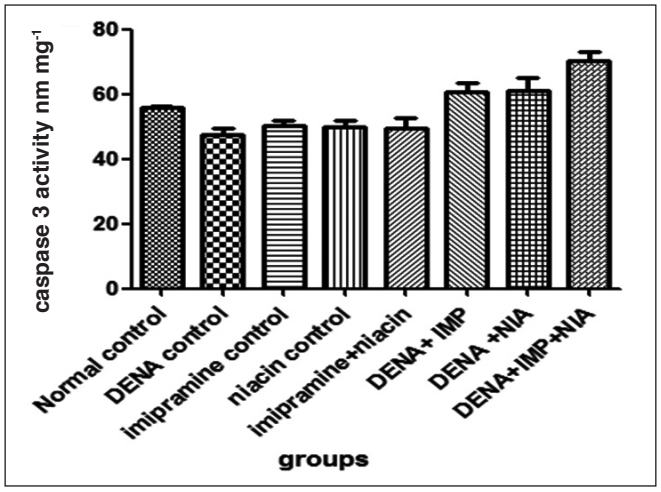

Fig. 2: Statistical data showing alteration in caspase-3 (antiapoptotic factor) activity

(p<0.01) and when compared to DENA + niacin control it was non-significant. Treatment with imipramine in combination with niacin significantly (p<0.001) decreased the elevated levels as compared to DENA control group (Table II).

Bilirubin (BIL)

BIL levels were elevated significantly (p<0.001) in DENA controls as compared to normal control group; DENA + imipramine control group reduced the elevated HDL significantly (p<0.001) and when compared to DENA+ niacin control, it was not significant. Treatment with imipramine in combination with niacin decreased significantly (p<0.001) the elevated levels as compared to DENA control group (Table II).

Alfa feto protein (AFP)

In DENA control group, the AFP levels were elevated significantly (p<0.001) as compared to normal control group; DENA control group when compared to DENA + imipramine 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 imipramine in combination with niacin decreased significantly (p<0.001) the elevated levels as compared to DENA control group (Table II).

Tumor growth

Three-dimensional ultrasound analyses revealed an almost 70% increase of tumor volume in DENA controls (Fig. 1). Of interest, imipramine and niacin combination inhibited tumor growth around 30% during the experimental period (Fig. 1).

Caspase-3 activity in liver

Our exploration also resulted in prominent alteration in caspase-3 (antiapoptotic factor) activity by established procedure of formation and detection of chromophore p-nitro anilide stated by Okhawa et al., Presence of chromophore by observing the changes in nitro anilide resulted in notable changes for the protocol. The activity was noted by ELISA at 405nm (Ohkawa et al; 1979) (Fig. 2)[34].

DISCUSSION

Any new molecule or drug or combination of drugs can only prove to have anticancer or hepatoprotective function if such drugs are able to normalize the physiological and anatomical architecture of the liver to its normal form which had been distorted by hepatotoxic or some other factor[16]. DENA is a liver specific carcinogen used in animals to produce hepatocarcinoma via free radicals and oxidative stress to liver[17]. DENA is known for its mutagenic activity causing mutation in DNA of hepatocytes leading to hepatocarcinogenesis[17,18]. Imipramine, a tricyclic antidepressant drug, reduces the cancer cell proliferation via Eag1 channels[19]. Further, it is well established that imipramine is responsible for apoptosis via mitochondrial and non-mitochondrial dependent pathways to establish its antitumor activity. It is well proved by researchers that in dose dependent manner (50μM) it is responsible for apoptosis in peripheral lymphocytes and cytotoxic T lymphocytes[11] along with activation of caspase by the hyper generation of intracellular reactive oxygen species

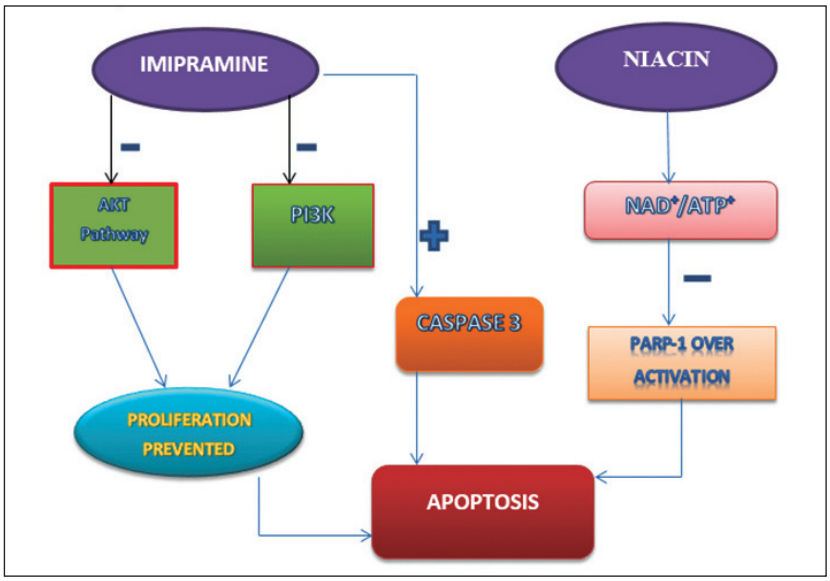

Fig. 3: Possible mechanism behind potent action of imipramine and niacin on hepatocellular carcinoma

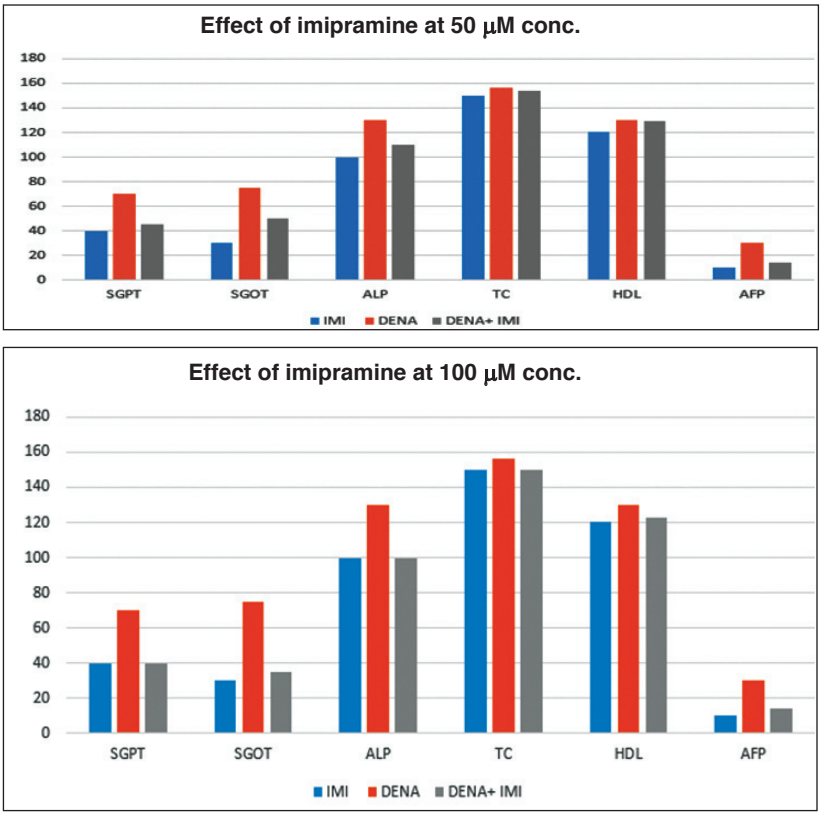

Fig. 4: *In vivo* effect of imipramine in 50*µ*M and 100 *µ*M concentration in liver profile of IMI control, DENA Control and DENA+ IMI control

(ROS). Niacin or vitamin B_3 is a precursor for the synthesis of nicotinamide adenine dinucleotide (NAD^+) and the phosphorylated derivative NADP[20]. Low intake of niacin is well recognized for high frequency of oral gastric and colon cancer[21]. In a trial conducted, one niacin and other micronutrient supplements decreased cancer incidence by 14% and mortality by 10%[22]. In cancer chemotherapy, NAD^+ level is generally depressed and becomes a leading cause for anorexia[23]. Numerous animal researches in rats have significantly correlated deficiency of Niacin with high risk of chemotherapeutic-induced secondary leukemia[24]. Inhibition of PARP-1 by nicotinamide has been shown to switch the mode of cell death from necrosis to apoptosis[25]. The balance between the ATP and NADP levels is the cellular and molecular switch oxylating between necrosis and apoptosis through oxidative stress[13, 26]. Nicotinamide is a precursor of NAD^+ ATP is an intracellular endogenous inhibitor of PARP-1, thus protecting the normal cell damage by various reactive oxygen species. Hence, the cellular fate in response to genotoxic DNA damage largely depends upon role of NAD^+. Our work through graphical abstract (Fig. 3) represents the mechanism underlying the therapeutic potential of imipramine and niacin in cancer prevention[26, 27].

The present study was an effort to evaluate the combined potential of imipramine and niacin in significant prevention of liver cancer. Both the drugs at individual level have potential anticancer activity, synergistic outcome of these drugs was more significant due to caspase 3 activation by them. Imipramine was monitored at two concentrations of 50 µM and 100 µM *in vivo* as an individual drug therapy and in combination with niacin *(ad libitium)* (Fig. 4). DENA induced hepatocellular damage had clearly been evident through marked elevation in serum SGPT, SGOT, ALP and a decreased level of HDL. These biochemical markers are indicators of tumor generation[28]. In our earlier studies, we have already observed that the elevated level of these enzymes is responsible for HCC[29]. The combination of imipramine and niacin significantly improves the level of these specific enzymes towards the normal level. Restoration of enzyme level by this combination, precisely SGPT, SGOT and ALP, has suggested the potential use of the study

combination in chemotherapy of HCC. The alteration i.e., increase in the level of alpha fetoprotein, is a standard marker for hepatocellular carcinoma which had also been markedly restored in therapeutic groups. The synergistic administration of imipramine and niacin has significantly reduced the elevated level of this protein in the therapeutic group. Our earlier research has further established AFP as a standard marker to confirm the decrease of its level in therapeutic group[30]. Our findings strongly suggest that the combination of imipramine and niacin significantly reduces the level of AFP[30,31].

Adding to the results of our study, increase in cholesterol levels in DENA induced animal models leading to its progression to fatty liver and cirrhotic condition in liver is well documented and explained by earlier research[32]. The increased cholesterol level is required for the formation of newly formed cancerous cells. The reduction in cholesterol and triglycerides level decreases the supply of these lipids for phospholipid membrane synthesis in cancerous cell though this effect was not seen when imipramine and niacin were used in their individual capacities; although combined regimen have definitely shown evident decrease in cholesterol levels[33]. The restructuring of liver cells by synergistic combination is a significant option for the treatment of liver cancer.

CONCLUSION

Data from the study suggest that imipramine and niacin combination possess chemo-preventive action. Imipramine (20 mg kg^{-1}) and niacin *(ad libitium)* combination at high doses suppresses the tumor lesions and markedly suppresses all elevated biochemical markers responsible for HCC via caspase 3 activated mechanism. This may open a new avenue for the patients who are under the influence of depression from liver or other associated cancers.

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