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IN VIVO TOXICITY COMPARISON STUDIES BETWEEN CAMPTOTHECIN AND DISULFIDE LINKED BIOTIN CONJUGATED CAMPTOTHECIN IN COLON TUMOR BEARING RATS

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Abstract:
Camptothecin is an important chemotherapeutic agent used in the treatment of several cancers and most commonly used as first line drug in treatment of colon cancer. However, it has several side effects including nephrotoxicity, hepatotoxicity, and ototoxicity. In *in vitro* experiments, several vitamins have shown antitumor protective effective against toxicity produced by chemotherapeutic drugs. Biotin is one such bioactive vitamin and various authors have reported that it has strong antioxidant and antitumor potential when used in conjugation with antitumor drugs. The present study was, therefore, carried out to explore the protective potential of CPT-SS-Biotin on DMH-induced hepatotoxicity and nephrotoxicity in colon tumor-bearing rats. Animals were divided into four groups: Group I: normal control, Group II: DMH treated, Group III: DMH+ CPT-SS-Biotin treated and Group IV: DMH+ standard camptothecin. Administration of conjugated CPT significantly ameliorated the toxicity caused by DMH as indicated by improved liver function tests, kidney function tests and hematological tests more efficiently in comparison to CPT alone. The same was also evident from the improvement in the histopathological changes in kidney and testis. Blood counts were also improved on administration of conjugate to DMH-treated rats. This article provides the evidence that antioxidant efficacy of biotin has beneficial effects on DMH-induced nephrotoxicity and hepatotoxicity.

Keywords: Antitumor, camptothecin, conjugation, biotin, toxicity

1. INTRODUCTION

Camptothecin (Fig, 1) is a naturally occurring anticancer agent, which was first isolated in 1966 from *Camptotheca acuminta*. It is a pentacyclic alkaloidal agent that possesses antitumor activity by inhibiting topoisomerase-I enzyme. CPT has been approved by FDA for its use in chemotherapy of colon cancer treatment either alone or in combination with other antitumor agents. Unfortunately, CPT on hydrolysis leads to carboxylate metabolite which induces severe hematological toxicity, nephrotoxicity, hepatotoxicity, hemorrhagic cystitis and diarrhoea in the patients.**[1]** The reason for its toxicity can be many but most commonly adopted mechanism is that it produces oxidative stress, which led to release of free radicals and thus causes toxicity. So, this problem can be minimized if any antioxidant molecules get conjugated with camptothecin i.e. a prodrug of camptothecin.

Rapidly proliferating tumor cells need various nutrients and vitamins for their growth and thus receptors of these molecules are overexpressed on the surface of tumor cells.**[2]**The most common micronutrients which are required by cancer cells in higher concentration are biotin**[3,4]**,vitamin B12**[5]**,riboflavin**[6]**and folic acid**[7].** Vitamin receptors are also helpful in imaging and tumor cell identification acting as biomarkers along with their drug delivery property.**[8,9]** All these molecules are essential for proliferation of all the cells, but rapidly growing tumor cells require them in higher concentration. Folate receptors were the first to get explored on this strategy using folic acid as tumor targeting moiety.**[10-13]**Similarly, biotin receptors are also overexpressed in various tumor cells e.g. leukemia (L1210FR), colon (Colo-26), ovarian (Ov2008, ID8), lung (M109), breast (4T1, JC, MMT06056), renal (RENCA, RD0995) and mastocytoma (P815) cell lines.**[14]**

Taking into consideration the potential clinical use of camptothecin and the numerous health benefits of biotin, the present study was aimed to test the hypothesis that disulfide linked biotin conjugated camptothecin derivative (CPT-SS-Biotin) (Figure 1) would ameliorate camptothecin-induced hepatotoxicity and nephrotoxicity allowing the clinical use of camptothecin in the treatment of various malignancies and minimizing its side effects. We evaluated the effect of CPT-SS-Biotin in the liver and kidney of colon tumor bearing wistar rats and *in vitro* studies were also conducted on cancer cell lines. To this end, we observed structural alterations, and histopathological changes caused by camptothecin and CPT-SS-Biotin on the liver and kidney of tumor-bearing rats. We also analyzed several hematological parameters along with liver function and kidney function markers for toxicity.

2. RESULT AND DISCUSSION

2.1Biochemical Parameters

2.1.1 Liver function test

According to literature, with the alterations in the level of some enzymes present in the serum like transaminases (AST and ALT) and phosphates (ALP), liver toxicity or damage can be easily predicted. In case of any hepatocytes damage, transaminase enzymes are released whereas, when phosphate enzymes levels are elevated it causes bile dysfunction resulting in intrahepatic or extrahepatic biliary obstruction. In case of DMH group levels of AST, ALT and ALP were highest and it can be because DMH metabolism in liver gave rise to free radicals, which in return attacks plasma membrane and intracellular enzymes get release into bloodstream because of this hepatic damage as cell wall got disrupted. Both treatment groups reduced these elevated levels, but conjugate proved to be more effective. CPT lowered them to some extent only and there was still liver damage. All these findings suggested that CPT-SS-Biotin have a better and effective safety profile than CPT. (Table **1**)

2.1.2 Kidney function test

For monitoring proper renal functions, biomarkers like creatinine clearance, urea and blood urea nitrogen (BUN) are examined and renal damage can be significant from their altered values than normal. Tubular injury can be predicted easily if elevated levels of these markers can be seen. Levels of BUN get elevated when there is extreme protein breakdown or nitrogen retention, while amino acid metabolism alterations causes increased urea levels. In case of CPT treated tubular injury can be seen from elevated levels of these markers but CPT-SS-Biotin significantly reduced all these parameters as the presence of biotin in conjugate can help in free radical scavenging and also disulfide linkage made it site specific. (Table **2**)

2.1.3 Hematological parameters

Chemotherapeutic drugs decrease levels of blood cells significantly when given for treatment of tumor and this effect is called as “Nadir effect”. Because of the toxicity of drug (CPT) on blood cells, lower levels of different blood parameters were observed in animal group treated with it. But

in case of CPT-SS-biotin conjugate these levels were almost near to normal range of blood count, which showed an improved effect and less toxicity than of free CPT. (Table **3**)

2.2 Histological analysis

The normal histo-architecture showing a prominent central vein and normal lobular structure in the liver of normal rats can be seen. The intact polyhedral structure was apparent, showing the plates of hepatocytes separated by the sinusoids. The nuclei and the cytoplasm showed usual staining. But these hepatic plates were disintegrated along with dilated sinusoids having increased intracellular gaps in case of DMH administered (control) rats and all this led to neutrophil infiltration. But this liver damage was less in both CPT and test compound administered rat liver tissues, however among both of them conjugate was well tolerated during the experimental period of 20 weeks (Figure **2**)**.**

Histopathology of kidneys in normal rats showed well-preserved glomerulus and intact tubular epithelial cell which confirmed kidneys from normal animals were completely healthy. DMH-treated groups showed mild tubulointerstitial injury. But it was absent in CPT and CPT-SS-Biotin treated groups which showed that both standard and test conjugate was well tolerated by the carcinogenic rats (Figure **3**).

3. CONCLUSION

In this work we compared the toxicities produced between a pure camptothecin drug and its conjugate in *in vivo* antitumor model and on *In vitro* cancer cell lines. From the results, it was concluded that anticancer activity of CPT-SS-Biotin was better than that of free CPT. It was because CPT-SS-Biotin reacted with intracellular thiol presented inside tumor cells and liberated CPT which made it more potent and selective; as it was the major drawback of parent drug to not differentiate between normal cells and cancer cells. *In vivo* studies on anti-tumor animal model depicted the ability of conjugate in tumor suppression more than the CPT alone, which was evident collectively from histological and serological studies. Conjugate was definitely able to minimize the side effects of parent drug like liver toxicity, renal damage. In summary, we can say that CPT drug conjugate can be recognized as a selective and specific drug delivery system which can accumulate inside tumor cells after preferentially taken up by them and then liberate active drug to achieve a high therapeutic efficacy and can be easily explored for clinical applications in future.

4. MATERIAL AND METHODOLOGY

According to the protocol approved by IAEC (MMCP/IAEC/16), all animal experiments were performed. From National Institute of Pharmaceutical Education and Research (NIPER), S.A.S. Nagar, Mohali, wistar Albino rats were obtained and according to Indian National Law on Animal Care and Use they were maintained in animal house. They had access to commercial pellet diet and water (*ad libitum)* and were kept in solid-bottomed polypropylene cages at 50% humidity, 25 ± 2°C of temperature keeping 12 h light/dark cycle. The 1,2 dimethyl hydrazine (DMH) was used for colon tumor induction by giving subcutaneous injection 15mg/kg/week for 5 weeks) in right shoulder. The administered dose given to the animals for conjugate testing was decided on the basis of toxicity studies performed in accordance with literature.

All the animals were divided into four groups (7 animals per group), with minimum weight differences; group I untreated animals group II control group (DMH), group III test group (DMH + CPT-SS-Biotin (25mg/kg), and group III standard group (DMH + CPT; CPT dose: 25mg/kg). Camptothecin was used as a standard drug and CPT-SS-Biotin conjugate as test compound to compare the toxicities produced by them. Blood samples were taken and animals were sacrificed to carry out their biochemical and histological studies (liver and kidney), respectively. **[15]**

4.1 Biochemical estimations:

4.1.1 Liver function test

Alkaline Phosphatase (ALP)

Alkaline Phosphatase enzyme is located inside the liver and bone and is utilized in protein breakdown. In case of liver damage or any disease, level of this enzyme gets elevated than normal. ALP activity was estimated in serum by using ENZOPAK ALP kit (CC1-ALK.02M, 50x1.1, 16AX02M) obtained from Reckon Diagnostic P. Ltd. (India). The kit is based on the hydrolysis of p-nitrophenyl phosphate into chromogenic compound p-nitrophenol. The rate of increase in absorbance of the reaction mixture at 405nm due to liberation of p-nitrophenol is directly proportional to the alkaline phosphatase activity. (Units obtained for ALP activity = IU/L). **[16]**

Alanine Transaminase (ALT)

ALT is another enzyme of liver responsible for providing energy to liver cells by conversion of proteins. In case of liver damage, levels of ALT in blood are increased because they get released into blood stream. ALT level was estimated in serum using ENZOPAK ALT kit (CC2-ALT.17N, 5x25 ml, B101311) obtained from Reckon Diagnostic Pvt. Ltd. (India). The kit was designed according to the described procedure of Henry et al., (1960). The assay included two step reaction in which pyruvate produced upon ALT or SGPT (Serum glutamate pyruvate transaminase) incubation was reduced by lactate dehydrogenase (LDH). The reduction was facilitated with the oxidation of NADH to NAD. The decrease in the absorbance of NADH at 340 nm was proportional to ALT activity. (Units obtained for ALT activity = IU/L). **[17]**

Aspartate Transaminase (AST)

Metabolism of amino acids takes place due to the presence of Aspartate Transaminase in liver. Same like ALP and ALT, there levels also get elevated from the normal in case of any liver or muscle damage or any disease. AST level was estimated in tissue homogenate and serum using ENZOPAK AST kit (CC2-AST.16N, 5x25 ml, B071927) obtained from Reckon Diagnostic P. Ltd. (India). The kit was designed according to the described procedure of Karmen et al., (1955). The assay included two step reaction in which oxaloacetate produced upon serum glutamate oxaloacetate transaminase (SGOT/AST) incubation was reduced by malate dehydrogenase (MDH). The reduction was facilitated with the oxidation of NADH to NAD. The decrease in the absorbance of NADH at 340 nm was proportional to AST activity. (Units obtained for AST activity = IU/L). [18]

4.1.2 Kidney function markers

Kidney function tests viz. Urea, creatinine, and blood urea nitrogen were performed and calculated with commercially available kits from Reckon diagnostics. (Creatinine; CC3-CRE.08M, 120 ml, B111229 and for Urea and BUN; CC2-UAB.019, 5x10 ml, B041128).

Creatinine

Due to any normal wear and tear to body muscles, a waste product comes out *i.e.* creatinine. According tpo age and body size levels of creatinine vary in bloodstream. Any increase in normal levels, indicate the presence of kidney disease or renal malfunction. With progression of kidney disease their levels get more elevated. (Units obtained for Creatinine = mg/ml). **[19]**

Urea

Metabolism of urea and nitrogen occurs in kidneys and it is also known as renal nitrogen metabolism and is required for healthy being. Any waste nitrogen gets converted into ammonia, and on further metabolism form urea which gets excreted. But in the presence of any kidney disease or renal failure there levels get elevated from normal, as kidney functions get impaired. (Units obtained for Urea and BUN = mg/ml). **[20}**

4.1.3 Hematological parameters

Blood sample (100µl) was collected from the retro-orbital plexus of mice using fine glass capillaries in sterile eppendorf tubes. Tubes containing blood were allowed to clot at 37°C for 4 hours. The clotted blood was centrifuged at 3000 rpm for 10 minutes and the upper clear layer (serum) was aspirated in another sterile eppendorf tube. Serum samples were stored at -80°C. (Units: Hemoglobin- g/dl, Platelets- x1000/µl, TLC- x10 counts, Neutrophils- %age, Lymphocytes- %age). **[21]**

4.2 Histopathological estimations

To evaluate the histological alterations, tissues from normal, control as well as from treatment groups were subjected to Haematoxylin and Eosin (H&E) staining. Tissue sections (liver and kidney) were washed with normal saline and were immediately fixed in 10% formalin for about 24 hours. After fixation, tissues were dehydrated in ascending grades of alcohol (30%, 50%, 70%, 90%, and 100%) for an hour each. Samples were then kept in alcohol + benzene mixture (1:1) for 30-45 min, followed by benzene for 30 min and then embedded in a mixture of benzene and paraffin wax (1:1) for 1 hour at 58-60ºC. Before proceeding for final embedding in the wax, the samples were immersed in pure molten wax with two changes of 3 hours each and were obtained as solid blocks, thereafter.5-7 microns thick paraffin sections were cut with the help of hand-driven microtome and then placed on clean glass slides.The sections so obtained were then dewaxed in xylene, rehydrated in descending series of ethanol (100%, 90%, 70%, 50%, and 30%),brought to water and stained in hematoxylin for 30 seconds. Slides were further treated with ascending series of ethanol (30%, 50%, and 70%) and were stained with alcoholic eosin for 1-2 min. The stained tissue sections were further differentiated with 90% ethanol and washed with absolute alcohol for 1 min each. The sections were finally cleared by rinsing the slides in xylene followed by mounting in DPX (Humanson, 1961). The tissue sections were analyzed under a light microscope (LEICA DM 3000). **[22]**

4.3 Statistical analysis

The results are expressed as mean ± SD (standard deviation) of seven animals in each group. For statistical significance, the data were analyzed using one-way ANOVA (Analysis of Variance). P values <0.05 were considered statistically significant.

AUTHOR CONTRIBUTIONS

Concept- Manu Sharma, Amardeep Kaur, Shikha Dhiman; Design – Manu Sharma, Amardeep Kaur, Shikha Dhiman ; Supervision – Manu Sharma; Resources – SERB-DST, Delhi and MM(DU), Mullana ; Materials – ; Data Collection and/or Processing – Manu Sharma, Amardeep Kaur, Shikha Dhiman; Analysis and/or Interpretation – Manu Sharma, Amardeep Kaur, Shikha Dhiman; Literature Search – Manu Sharma, Amardeep Kaur, Shikha Dhiman; Writing – Manu Sharma, Amardeep Kaur, Shikha Dhiman; Critical Reviews – Manu Sharma, Amardeep Kaur, Shikha Dhiman.

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CONFLICT OF INTEREST

No conflicts of interest declared by authors.

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TABLES

Table 1: Effect of DMH, DMH+CPT, DMH+CPT-SS-Biotin on liver function after 20 weeks of treatment

Group/Parameter	AST	ALT	ALP
Normal	39.3±0.88	45.92±0.99	41.8±0.56
DMH	64.10±0.66	69.08±1.40	172.37±1.12
DMH+CPT-SS-Biotin	47.94±1.95	57.57±1.69	83.81±1.02
DMH+CPT	60.41±1.01	63.64±0.77	121.14±1.35

Units: IU/L; Data is expressed as Mean ±SD (n = 7). Data is analyzed using one-way ANOVA with keeping $p \leq 0.05$

Table 2: Effect of DMH, DMH+CPT, DMH+CPT-SS-Biotin on kidney function after 20 weeks of treatment

Group/Parameter	Urea	BUN	Creatinine
Normal	26.45±1.2	13.6±0.85	3.41±0.55
DMH	55.32±2.05	29.08±1.25	9.11±0.73
DMH+CPT-SS-Biotin	32.70±1.96	15.08±0.84	6.87±0.79
DMH+CPT	43.34±1.41	20.40±1.09	7.68±0.66

Units: Urea- mg/dl; BUN- mg/dl; Creatinine- mg/dl; Data is expressed as Mean ±SD (n = 7). Data is analyzed using one-way ANOVA with keeping $p \leq 0.05$

Table 3: Effect of DMH, DMH+CPT, DMH+CPT-SS-Biotin on blood count after 20 weeks of treatment

Group/Parameter	Hb	Platelets	TLC	Lymphocytes	Neutrophils
Normal	13.1±0.5	64.27±1.82	828.14±1.77	71.71±2.28	35.42±2.37
DMH	9.85±0.98	70.31±2.86	968.42±26.85	80.20±4.05	30.81±1.91
DMH+CPT	8.51±0.51	61.74±2.60	599.14±8.97	71.57±4.55	34.05±1.52
DMH+CPT-SS-Biotin	12.48±0.70	67.85±1.84	630.71±5.82	79.66±1.59	31.57±2.25

Units: Hemoglobin- g/dl, Platelets- x1000/ul, TLC- x10 counts, Neutrophils- %age, Lymphocytes- %age; Data is expressed as Mean ±SD (n = 7). Data is analyzed using one-way ANOVA with keeping $p \leq 0.05$

FIGURES

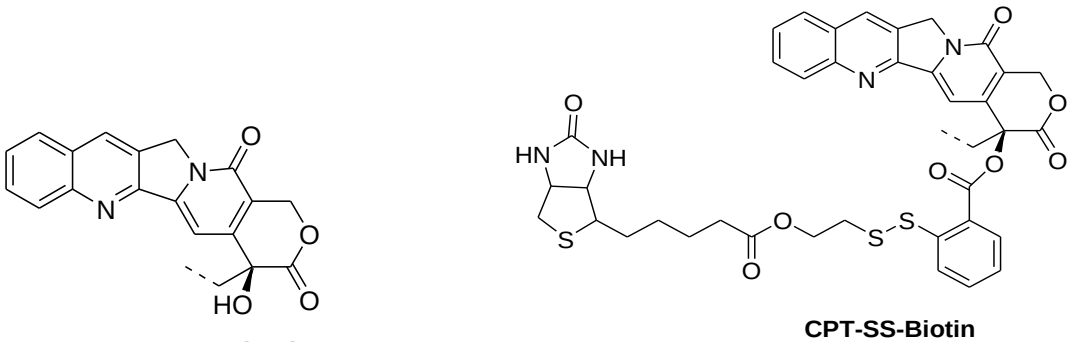

Figure 1: Structure of camptothecin and disulfide linked biotin conjugated camptothecin

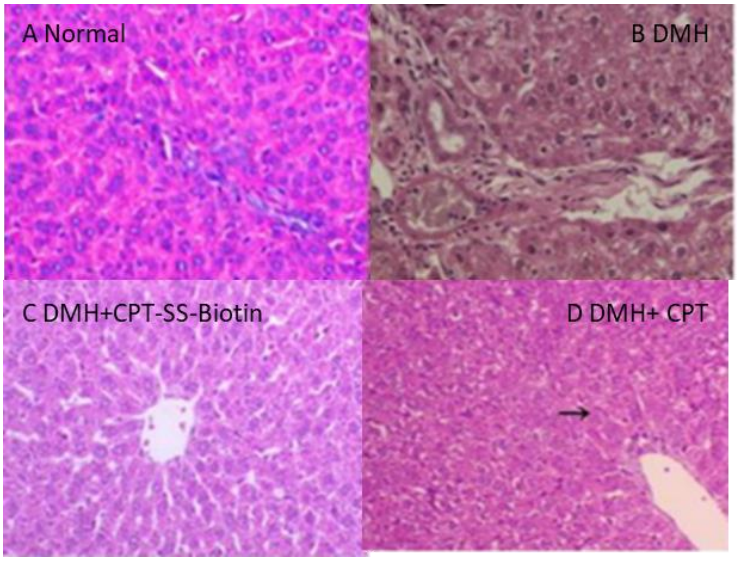

Figure 2: Histological observations of the liver induced with DMH and the effect of standard drug and conjugate treatment. (a) Normal morphology can be seen in liver section of group-I as central portal vein can be seen. (b) Sinusoidal dilation, necrosis and congestion seen in rats exposed to DMH (group 2) in liver section. (c) Conjugate (group 3) treated rats with mere cell infiltration have near to normal histoarchitecture (d) Hyperplasia and cell infiltration shown in liver of rats administered with camptothecin alone along with DMH.

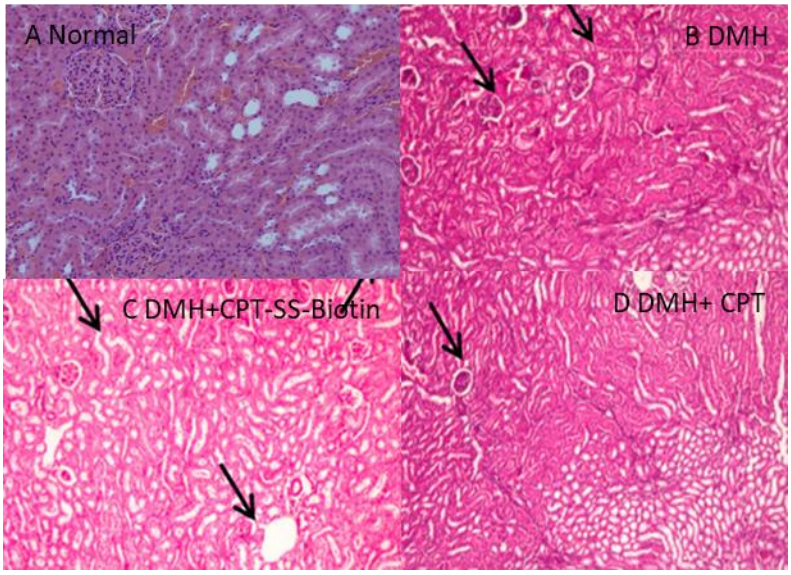

Figure 3: Effect of DMH, camptothecin, and CPT-SS-Biotin on histoarchitecture of kidney tissue after 20 weeks of treatment [Bowman's capsule, Tubular regions, glomerular congestion and degeneration and degeneration in tubular cells (TR)]