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Research Article

The Effect of Substituted Thiopyrimidine Acyclic Nucleosides and Their Thioglycoside Analogs as Novel Anti-cancer Agents Targeting Metastasis and Angiogenesis on N-Nitrosodiethylamine Induced Hepatocellular Carcinoma in Rats

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ABSTRACT

Hepatocellular carcinoma is a serious healthcare problem worldwide because of its increasing morbidity and high mortality rates. In our previous work new thiopyrimidine acyclic nucleosides and thioglycoside analogs 3a-c, 4a-c, 6a,b and 7a,b were synthesized and identified. Furthermore, the antitumor activities of all prepared compounds were evaluated *in vitro* against Ehrlich ascites carcinoma cells and some compounds (2, 3b, 3c, 4a and 4c) showed promising anticancer effect. The present study aimed to investigate the anticancer effects of the prepared compounds (2, 3b, 3c, 4a and 4c) by studying their ability to inhibit, reverse or restrict the development of cancer through inhibiting the metastasis and angiogenesis of tumor in N-Nitrosodiethylamine induced hepatocarcinogenesis in rats. To elucidate the mechanism by which these compounds exert their antitumor activities in the animals-bearing tumor the following parameters were determined (after determination their median lethal dose, LD_{50}) including aspartate and alanine aminotransferases, alkaline phosphatase, total bilirubin as liver function test; hepatic tyrosine kinase and cytochrome P450 2E1 as markers for tumor progression; vascular endothelial growth factor (VEGF) and total sialic acid (TSA) as markers of angiogenesis; heparanase and elastase as markers of metastasis. Liver histopathological analysis was also evaluated. The results revealed that, carcinogenic rats recorded drastic elevation in all parameters under investigation which confirmed by histopathological distortion in the tissue organization with hyperchromatism, hyperplasia, proliferating hepatocytes. Compounds supplementation at 1/10 of the LD_{50}, significantly reversed (improved) the biochemical and histopathological changes induced by NDEA in the order of 3c > 3b > 4a > 2> 4c. From the foregoing results we can concluded that, the tested compounds especially 3c and 3b may be potent anticancer agents for inclusion in modern clinical trials after more investigations on higher animals.

Keywords: Hepatocellular carcinoma, anticancer, thiopyrmidines, angiogenesis, metastasis.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the major health burdens worldwide [1]. It accounts for about 80-90% of all liver cancers [2]. HCC affects around twice as many men as women and is more common in those above the age of 40 [3]. It represents the third most common cause of cancer death in the world. It results in 598,000 deaths per year worldwide and fifth most common cancer worldwide. Because of its poor prognosis, this number of deaths is almost the same as the number of cases being diagnosed each year (626,000) [4]. Each year, HCC is diagnosed in more than half a million people worldwide [5]. Most of the burden of the disease (85%) is borne in developing countries [6].

In Egypt, HCC is third among cancers in men with > 8000 new cases predicted by 2012 [7]. HCC was reported to account for about 4.7% of chronic liver disease patients. In 2005 a remarkable increase from 4 to 7.2% was reported over a decade [8,9]. Egypt has the highest prevalence of HCV in the world with 20% of the population infected and seven million with chronic HCV liver disease [10]. Up to 90% of HCC cases in the Egyptian population were attributed to HCV. Once cirrhosis has developed, retrospective studies have suggested that patients will develop either hepatic decompensation or HCC at a rate of 2% to 7% per year [11]. The burden of HCC has been increasing in Egypt with a doubling in the incidence rate in the past 10 years. This has been attributed to several biological (e.g. hepatitis B and C virus infection) and dietary pollution (e.g. aflatoxin B1) [12]. Other factors such as drugs, medications [13], occupational exposure to chemicals such as pesticides, hemochromatosis and endemic infections in the

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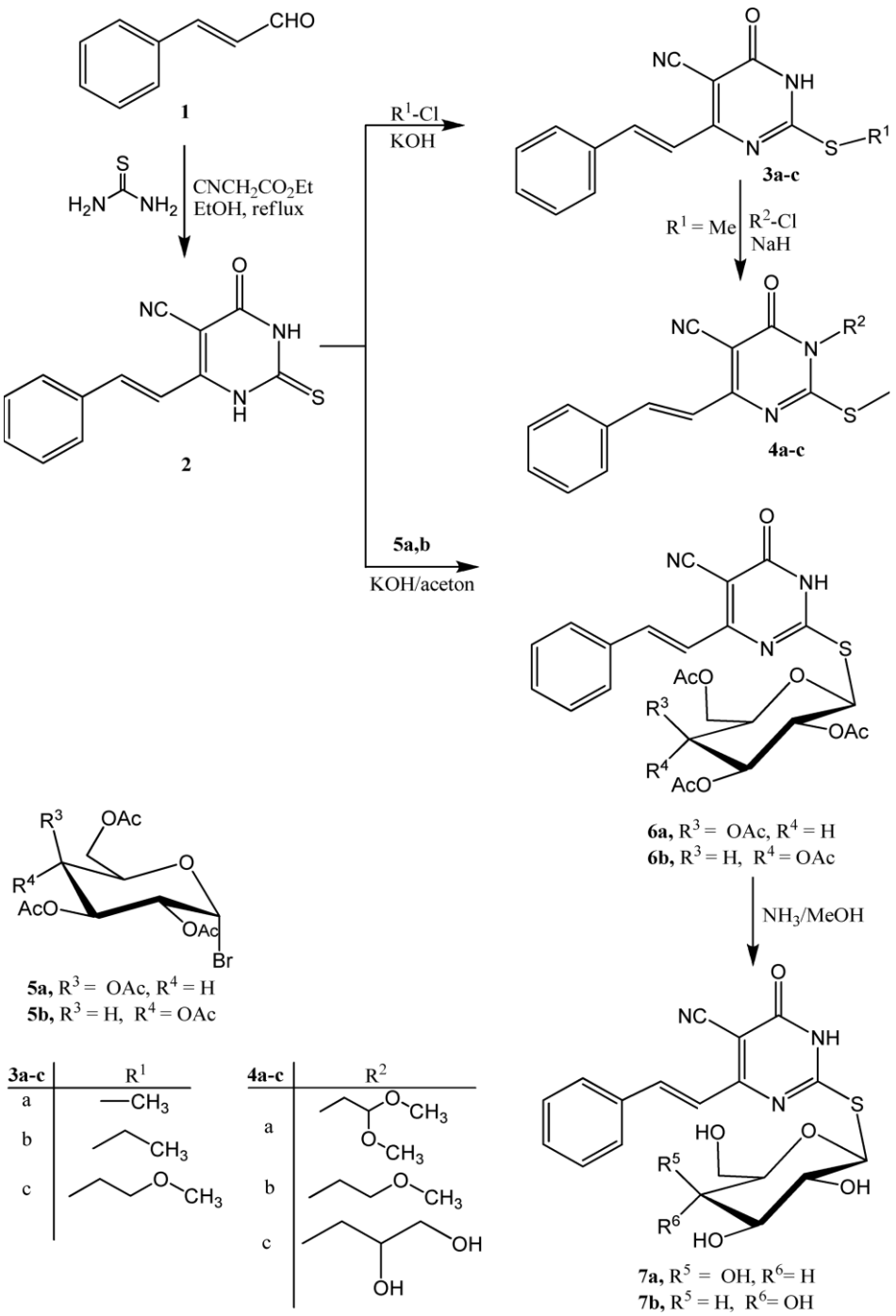

Figure 1: Synthesis route of substituted thiopyrimidine derivatives 2–7.

Table 1. In *vivo* the median lethal dose (LD_{50}) of the synthesized fused pyrimidine and thiopyrimidine nucleoside analogs

Compounds	LD_{50} µg/kg b.w.
2	132
3b	108
3c	80
4a	120
4c	160

community, such as schistosomiasis, may have additional roles in the etiology or progression of the disease [14,15.]

Pyrimidines have been recognized as important heterocyclic compounds due to their diverse biological activities such as Tie-2 kinase inhibitors, HIV-1 inhibitor, antimalarial, secretive adenosine A1 receptor antagonist, antibacterial, anticancer, analgesic, cardiovascular,and antiallergic activities [16]. The thio analogues of pyrimidine bases, including 2-thiouracil, are minor components of *t*-RNA. Their *S*-, *N*- or *S,N* -disubstituted analogs have shown therapeutic properties, especially antiviral, antithyroid and antitumor activities due to their incorporation into polynucleic acids and therefore act as potential inhibitors of protein and polynucleic acid syntheses. On the other hand, nucleoside analogs are structurally, metabolically, and pharmacodynamically related agents that have diverse biological actions and

Table 2. Effect of the synthesized fused pyrimidine and thiopyrimidine nucleoside analogs on serum AST, ALT, ALP activities and bilirubin level in different studied groups.

Groups	AST (U/L)	ALT (U/L)	ALP (U/L)	Bili (mg/dl)
Control	56.00±5.20	38.00±3.70	110.00±9.30	1.40±0.11
NDEA	110.00±7.80[a]	95.00±7.00[a]	215.00±15.70[a]	2.90±0.20[a]
2	88.70±7.90 [a,b]	80.00±7.0 [a,b]	190.00±16.0[a,b]	2.70±0.21 [a]
3b	65.40±7.4 [a,b]	63.00±6.11[a,b]	120.80±13.4[a,b]	1.88±0.21 [a,b]
3c	58.00±5.2 [b]	42.00±4.00 [b]	100.00±11.0 [b]	1.50±0.11 [b]
4a	85.00±7.80 [a,b]	75.00±5.90 [a,b]	175.0±15.0 [a,b]	2.50±0.18 [a,b]
4c	95.40±8.60 [a]	85.00±8.14 [a]	190.90±17.20[a]	2.75±0.22 [a]

Results expressed as Mean ± S.E.
[a] Significantly different from normal control at $p < 0.05$.
[b] Significantly different from NDEA - treated rats at $p < 0.05$.

therapeutic effects including antiviral and antitumor activities. Furthermore, the glycosylthio heterocycles and acyclic nucleoside analogues including modifications of both the acyclic glycon and aglycon parts have stimulated extensive research as biological inhibitors [16].

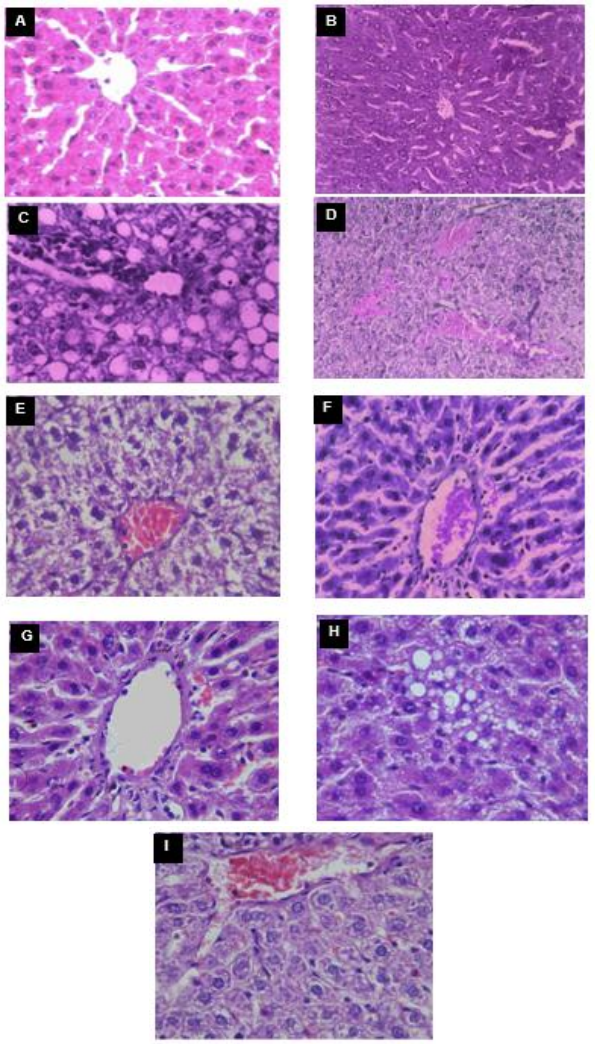

Figure 2: Represents the histological changes in liver of control (A); NDEA-treated rats (B-D) and cancer-bearing rats after the treatment with the prepared new thiopyrimidine acyclic nucleosides and thioglycoside analogs. Histological sections were prepared from compound 2 (E), compound 3b (F), compound 3c (G), compound 4a (H), and compound 4c (I). Sections were stained with hematoxylin and eosin (original magnification x250).

It is now well established that solid tumor growth is critically dependent on the growth of new vessels from preexisting blood vessels surrounding the tumor, a process called angiogenesis [17]. On the basis of this finding, the development of drugs that inhibit angiogenesis has become an attractive approach to cancer therapy [18]. In addition, metastasis of cancer cells to distant sites is one of the major deciding factors in cancer outcome. In fact, prognosis of cancer is mainly determined by the invasiveness of the tumors and its ability to metastasize. There is a cascade of events leading to the metastasis of tumors. These include separation from the primary site, circulation through blood or lymph, adhesive to the basement membrane (composed mainly of heparan sulfate, elastin, and collagen), invasion and proliferation at distant sites [19]. Any compound which can inhibit one of the steps in the cascade will be useful in the inhibition of tumor metastasis and tumor growth.

Owing to the above facts, the aim of the present work is to study the anticancer effect of previously synthesized thiopyrimidine acyclic nucleosides and thioglycoside analogs which gave good anticancer effect *in vitro* (2, 3b, 3c, 4a and 4c) [16] for inhibiting, reversing or restricting the development of cancer and inhibition of metastasis and angiogenesis of tumor in the experimental animals carrying liver cancer induced by N-Nitrosodiethylamine (NDEA) by studying different biochemical and histological investigation methods.

MATERIALS AND METHODS

Animals

The animal care and handling was done according to the guidelines set by the World Health Organization, Geneva, Switzerland, and according to approval from the ethics committee for animals care at the National Research Centre, Egypt (ethic No. 10-230). Adult male Sprague-Dawley rats (180±20 g, body weight), were purchased from the animal house of National Research Centre, Egypt. The animals were housed under standard laboratory conditions (constant temperature 25-27 °C, with 12 h light/dark cycle) during the experimental period. The rats were provided with tap water and commercial diets. The

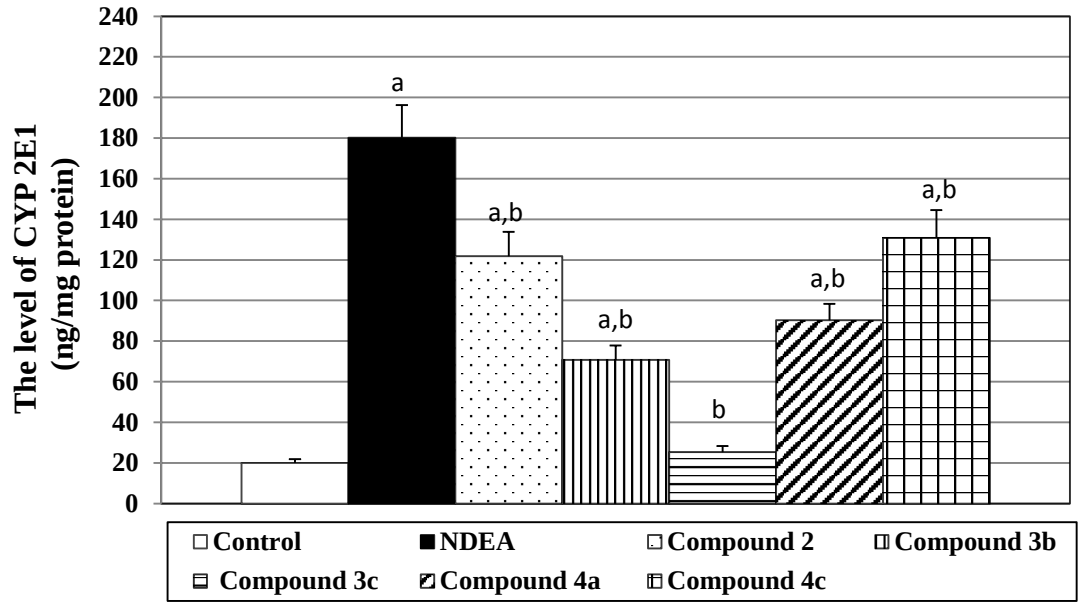

Figure 3: Effect of treatment with the synthesized thiopyrimidine acyclic nucleosides and thioglycoside analogs (2, 3b, 3c, 4a and 4c) on the level of hepatic Cytochrome P450 2E1 (CYP 2E1). Data were expressed as mean ± S.E., [a] and [b] is significant difference from control and NDEA - treated rats respectively at ($p < 0.05$).

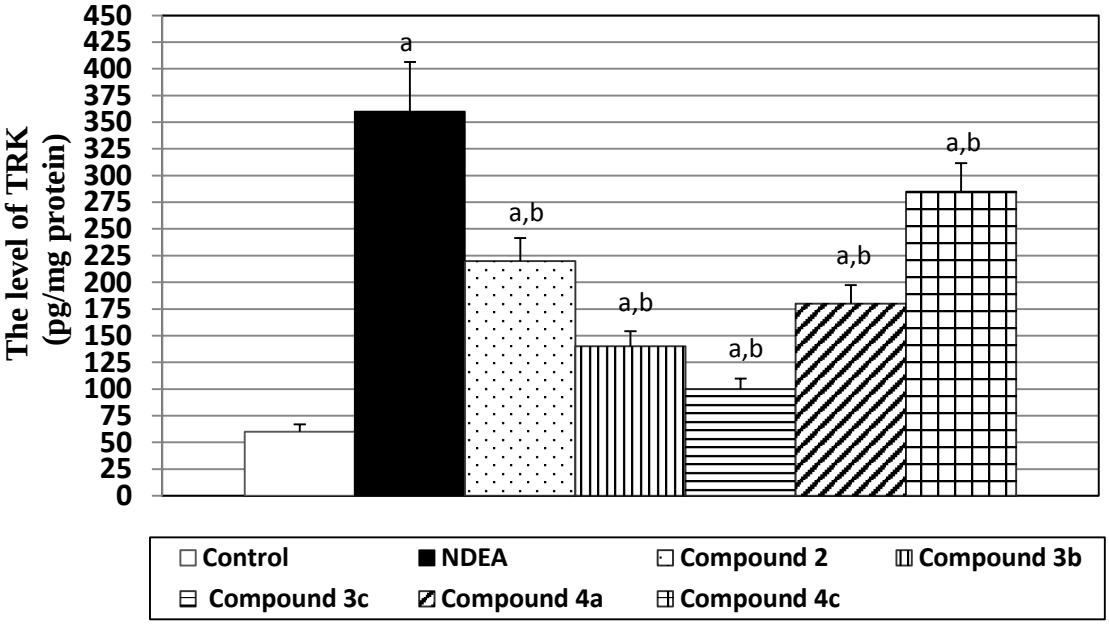

Figure 4: Effect of treatment with the synthesized thiopyrimidine acyclic nucleosides and thioglycoside analogs (2, 3b, 3c, 4a and 4c) on the expression of hepatic tyrosine kinase (TRK). Data were expressed as mean ± S.E., [a] and [b] is significant difference from control and NDEA - treated rats respectively at ($p < 0.05$).

rats were acclimatized to laboratory condition for 10 days before commencement of the experiment.

In Vivo the cytotoxicity of prepared thiopyrimidine acyclic nucleosides and thioglycoside analogs

The median lethal doses (LD_{50}) of the previously prepared thiopyrimidine acyclic nucleosides and thioglycoside analogs (2, 3b, 3c, 4a, and 4c) (Figure 1) was determined *in vivo* according to Ghosh [20]. Briefly, adult male Sprague-Dawley rats were randomly divided into groups of 10 per group. Each group was separately administrated once daily for a period of 4 weeks with doses ranging from 0-500 µg/kg b.w. of the compounds intraperitoneal (i.p.) in a value of 1 mL/kg body weight. Control animals received the vehicle alone. The animals were then provided with food and water immediately after the administration. The mortality of the animals was observed up to one month post-treatment. The

LD_{50} of the prepared compounds was calculated by using a computer program of probit analysis.

Experimental design

N-Nitrosodiethylamine (NDEA) and carbon tetrachloride (CCl_4) were purchased from Sigma Chemical Co. (St.

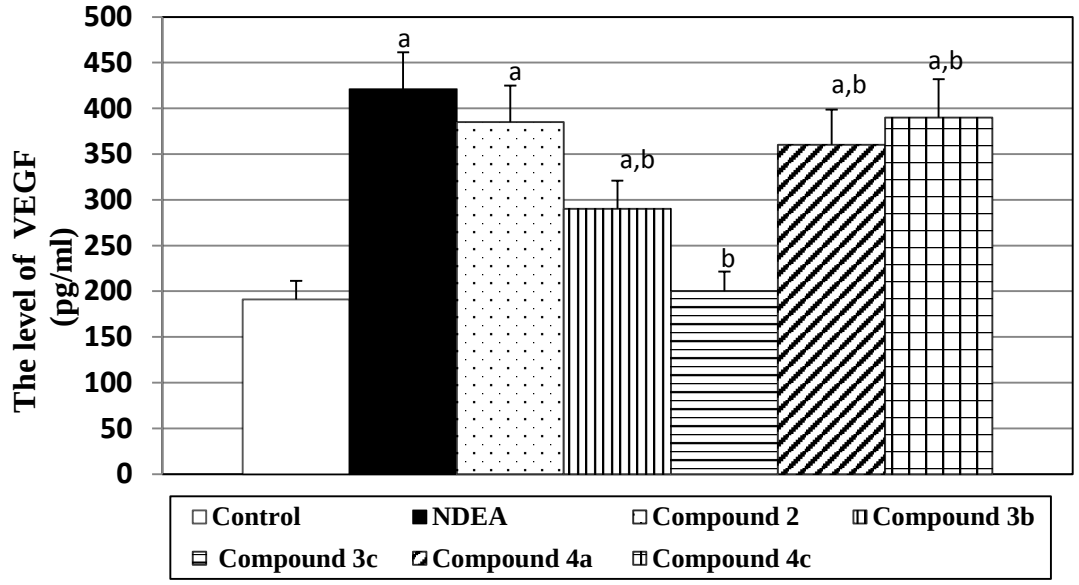

Figure 5: Effect of treatment with the synthesized thiopyrimidine acyclic nucleosides and thioglycoside analogs (2, 3b, 3c, 4a and 4c) on the level of VEGF. Data were expressed as mean ± S.E., [a] and [b] is significant difference from control and NDEA - treated rats respectively at ($p < 0.05$).

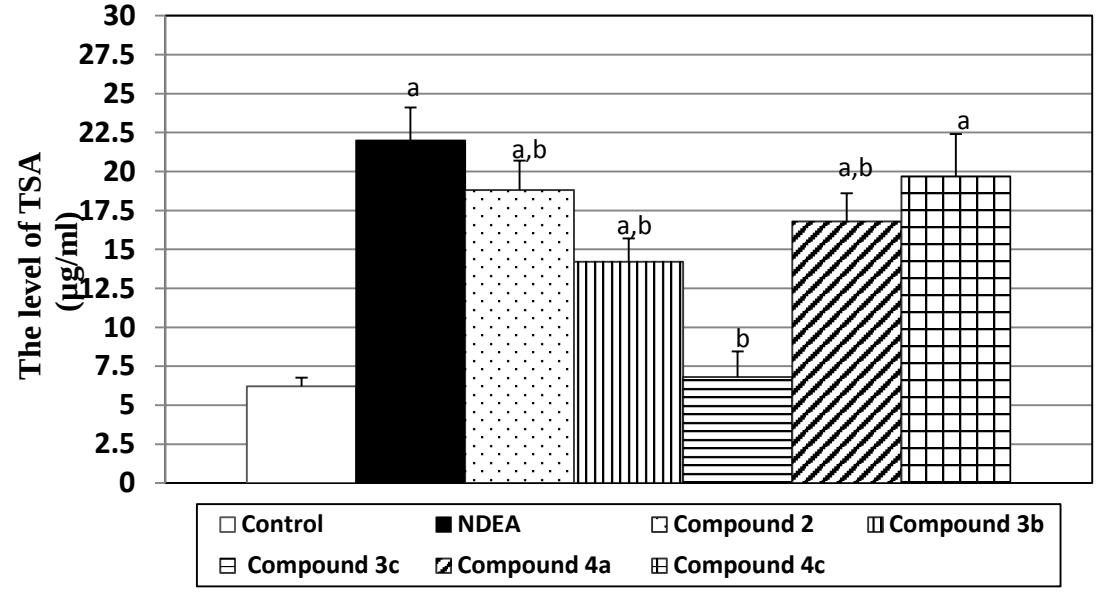

Figure 6: Effect of treatment the synthesized thiopyrimidine acyclic nucleosides and thioglycoside analogs (2, 3b, 3c, 4a and 4c) on the level of sialic acid (TSA). Data were expressed as mean ± S.E., [a] and [b] is significant difference from control and NDEA - treated rats respectively at ($p < 0.05$).

Louis, MO, USA). NDEA was dissolved in saline and injected in a single dose (200 mg/kg, i.p.) to initiate hepatic carcinogenesis, while CCl_4 was used in a single dose (2 mL/kg) by gavage as 1:1 dilution in corn oil to stimulate liver cell proliferation and regeneration [21]. The experiment continued for 32 weeks.

Adult male Sprague-Dawley rats were divided into groups with 10 animals in each group. Group 1 (untreated control group): animals were fed on a standard diet and given water throughout the course of the experiment. Group 2 (NDEA treated group): Rats were injected with a single dose of NDEA (200 mg/kg, i.p.) and 2 week later received a single dose of CCl_4 (2 mL/kg) by gavage as 1:1 dilution in corn oil for 32 weeks. Group 3 (NDEA and synthesized compounds group): Rats from Group 2 were treated daily with synthesized compounds (2, 3b, 3c, 4a and 4c) by i.p. treatment at dose of 1/10 of their LD_{50} values and continued for 32 weeks.

At the end of the treatment protocol (32 weeks), animals were anesthetized with ether and blood samples were drawn from the orbital venous plexus. Serum was separated by centrifugation for 5 min at 1500 g and stored at -20°C until analysis.

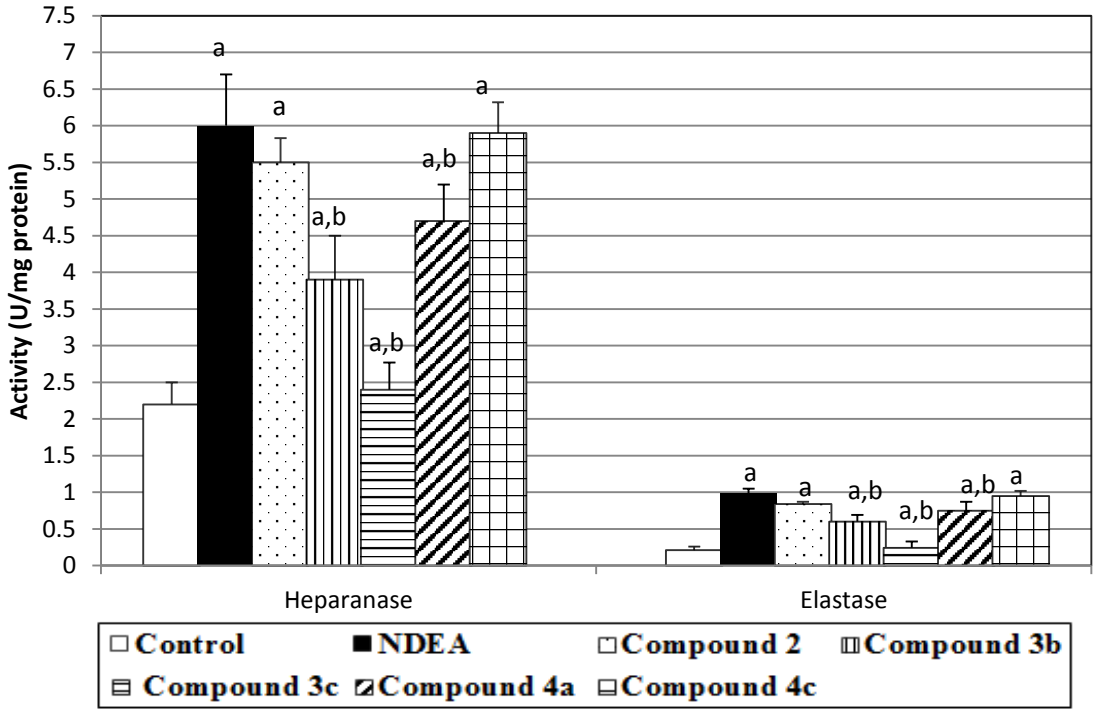

Figure 7: Effect of treatment with the synthesized thiopyrimidine acyclic nucleosides and thioglycoside analogs (2, 3b, 3c, 4a and 4c) on the activity of heparanase and elastase. Data were expressed as mean ± S.E., [a] and [b] is significant difference from control and NDEA - treated rats respectively at ($p < 0.05$).

All animals were sacrificed by decapitation and their livers were rapidly excised, weighed, washed with saline and blotted with a piece of filter paper. Portion of the liver was immediately fixed in 10% formalin for histological analysis according to Conn *et al.* [22] using a standard method of hematoxylin and eosin. Another portion of liver was homogenized using a Branson Sonifier (250 VWR Scientific, Danbury, Conn., USA) in cold sucrose buffer (0.25 M). All the investigation will carry out in fresh 10% homogenate. The freshly prepared homogenates were then centrifuged at 30,000 xg for 30 min at 4°C to obtain the supernatant, which used for biochemical assays and the protein level was determined as described by Lowry *et al.* [23].

Aspartate and alanin-aminotransferase (AST and ALT), alkaline phosphatase (ALP) activities as well as the level of total bilirubin (Bili) were determined spectrophotometrically according to the manufacturer's instructions, using reagent kits obtained from Biomerieux (France).

Cytochrome P450 2E1 (CYP 2E1) assay

The effect of synthesized compounds on the expression of Cytochrome P450 2E1 (CYP 2E1) was determined in tissue homogenates based on a double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) of Cytochrome P450 2E1 kit purchase from Cloud-Clone Crop. (Houston, TX 77082, USA). The concentration of CYP 2E1 in the samples is determined by comparing the O.D. of the samples to the standard curve.

Tyrosine kinase assay

The effect of synthesized compounds on the expression of tyrosine kinase (TRK) was determined in the tissue homogenates based on a double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) of tyrosine kinase kit purchase from Glory Science Co., Ltd (Del Rio, TX 78840, USA) according to the manufacturer's instructions. The chroma of color and the concentration of the human TRK of sample were positively correlated and the optical density was determined at 450 nm. The level of TRK in samples was calculated as triplicate determinations from the standard curve

Estimation of VEGF concentration

VEGF concentration in the serum was determined using ELISA kit obtained from Koma Biotech Inc., Korea. This assay depends on binding VEGF antigen to a specific immobilized antibody. The formed immune complex binds to avidin-peroxidase conjugate, and a color developed in proportion to the amount of VEGF bound which was measured at 450 nm.

Total sialic acid (TSA)

TSA was estimated in the serum by periodate-resorcinol microassay as described by Surangkul *et al.* [24]. The principle of this method is based on a periodate-resorcinol reaction with sialic acid found in the sample. The absorbance was measured at 620 nm immediately by a microtiter plate reader then N-acetyl neuraminic acid standard curve was used to calculate TSA concentration.

Determination of heparanase (HPSE) activity

Heparanase activity in tissue homogenates was determined based on a double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) of heparanase kit purchase from Glory Science Co., Ltd (Del Rio, TX 78840, USA) according to the manufacturer's instructions. The kit assay HPSE activity in the sample, use purified HPSE to coat

mictotiter plat wells, make solid-phase antibody, then add HPSE to wells, combined HPSE which with enzyme labeled, become antibody-antigen-enzyme-antibody complex, after washing completely, add substrate solution, the color change is measured spectrophotometrically at a wavelength of 450 nm. The activity of HPSE in the samples is then determined by comparing the absorbance of the samples to the standard curve. The activity was determined as U/mg protein.

Estimation of elastinolytic activity

The elastase activity is determined in the tissue homogenates by its catalytic effect on the *N*-succinyl-trialanyl-*p*-nitroanilide substrate releasing *p*-nitroaniline which is measured photometrically at 405 nm [25]. The elastase activity was determined as U/mg protein.

Statistical analysis

The results were reported as Mean ± Standard error (S.E.). Statistical differences were analyzed by one way ANOVA test followed by student's *t* test wherein the differences were considered to be significant at $p < 0.05$.

RESULTS

In Vivo the cytotoxicity of the synthesized compounds

The LD_{50} (the median lethal dose resulted in 50% mortality of the animals) of each compound was determined. Compounds 2, 3b, 3c, 4a, and 4c showed marked acute toxicity (Table 1). The concentrations required by 2, 3b, 3c, 4a, and 4c for 50% mortality of the animals were found to be 132, 108, 80, 120 and 160 µg/kg body weight, respectively. From the foregoing results it is clear that compounds 3b and 3c were the best compounds in this series.

The effect of the synthesized fused pyrimidine and thiopyrimidine nucleoside analogues on liver tissue

NDEA-treated rats showed significant ($p<0.05$) increase in serum AST, ALT and ALP activities, along with significant ($p<0.05$) increase in total bilirubin level compared to control. The administration 2, 3b, 3c, 4a, and 4c at a dose of 1/10 of the LD_{50} values in the NDEA-treated rats resulted in normalization in AST, ALT and ALP activities as well as the total bilirubin level compared to NDEA-treated group (Table 2). The tested compounds has resulted in decreasing in the level of liver function test follows the order 3c > 3b > 4a > 2> 4c. It is clear that, 3b and 3c were the best compounds in this series.

In this study histological examination of rat liver sections was consistent with the results obtained from biochemical studies. Liver of control animals as presented in (Fig. 2a) revealed normal architecture of hepatic strands around the central veins. The liver showed intact hepatocytes with normal sinusoids in between. The hepatic cells are polygonal in shape with one or two rounded nuclei. Liver of rats treated with NDEA alone showed distortion in the tissue organization with hyperchromatism, hyperplasia, proliferating hepatocytes (Fig. 2b), both hepatic and portal with significant tumor thrombi within portal vessels, tumor cells are slightly larger have more irregular nuclei and numerous mitotic figures with malignant nuclei (Fig. 2c). Some section showed megalocytosis, hyperchromatic nuclei as well as nuclear vacuolization and nuclear prominence, dissolution of hepatic cords which appeared as empty vacuoles aligned by strands of necrotic hepatocytes (Fig. 2d). Liver of the NDEA-rats treated with compound 2 showed diffuse hepatocyte necrosis with congested central vein (Fig. 2e). Liver sections from rats treated with compound 3b exhibited improved the hepatocellular architecture with more regular and less altered hepatocytes when compared to group treated with NDEA alone (Fig. 2f). Liver sections from rats treated with compound 3c showed normal appearance with normal appearing hepatocytes cords (Fig. 2g). Liver section from rats treated with compound 4a exhibited moderate ballooning degeneration of hepatic cells; focal fatty change of hepatocytes (Fig. 2h). Liver sections from rats treated with compound 4c showed areas of aberrant hepatocellular congested central vein with moderated focal hepatocyte necrosis. Severe infiltration of portal tract by inflammatory cells Hepatocytes showed ground glass appearance (fig. 2i).From the foregoing results it is clear that, there are improvement in the histological changes in the order of 3c > 3b > 4a > 2> 4c.

Effect of prepared compounds on cytochrome P450 (CYP) 2E1 and tyrosine kinase expression

The effect of the prepared compounds on both CYP 2E1 and TRK which implicated in cancer growth was illustrated in figure 3 and 4. The expressions of both CYP 2E1 and TRK were significantly increased in NDEA-treated as compared to normal control. While the administration of compounds 2, 3b, 3c, 4a, and 4c at a dose of 1/10 of the LD_{50} values in the NDEA -treated rats resulted in significantly inhibitory potential against both CYP 2E1 and TRK for all the compounds comparing with the NDEA group. Compounds 3c and 3b were the most potent inhibitor against CYP 2E1 and TRK expression.

Effect of prepared compounds on VEGF and TSA

The effect of the prepared compounds on VEGF and TSA as markers of angiogenesis was illustrated in figure 5 and 6. In this study, it was found that the levels of both VEGF and TSA in the NDEA-treated group was very highly significant increase as compared to control group while the treatment with 2, 3b, 3c, 4a, and 4c in the NDEA-treated rats, causes decrease in the level of VEGF and TSA as compared with NDEA-treated rats, while the VEGF and TSA level showed significant decrease in compounds 3c and 3b -treated group reaching to its control level.

Effect of prepared compounds on heparanase and elastase activity

In the present study the activity of heparanase and elastase as marker for metastasis of tumor was investigated, the results showed that the activity of heparanase and elastase enzymes was very highly significantly increased in NDEA-treated group. The treatment with 2, 3b, 3c, 4a, and 4c in the NDEA -treated rats, resulted in decrease in the activity of heparanase and elastase enzymes as compared with NDEA-treated group especially compounds 3c and 3b (Figure 7).

DISCUSSION

In our previous work new thiopyrimidine acyclic nucleosides and thioglycoside analogs 3a-c, 4a-c, 6a,b and

7a,b were synthesized starting with cinnamaldehyde, ethyl cyanoacetate and thiourea in ethanol. Furthermore, the antitumor activities of all prepared compounds were evaluated in vitro against Ehrlich ascites carcinoma cells and some compounds (2, 3b, 3c, 4a and 4c) showed promising anticancer effect [16].

Experimental liver cancer in rodents induced by NDEA, an environmental and dietary hepatocarcinogen [26], has been considered as one of the best characterized experimental models of HCC, allowing the screening of potential anticancer compounds on various phases of neoplastic transformation and development [27]. NDEA-induced preneoplastic foci and preneoplastic and neoplastic nodule formation in rodents closely mimics HCC development in humans [28]. Recently, a cross-species comparison of gene expression patterns has established that NDEA-induced liver tumors in rodents closely resemble a subclass of human HCC [28], which allows extrapolating potential chemopreventive effects of a candidate agent in clinical setting.

In the present study, we have investigated the preventive effect of prepared new thiopyrimidine acyclic nucleosides and thioglycoside analogs including 2, 3b, 3c, 4a and 4c on the appearance of early hepatic preneoplastic events, utilizing a two-stage model of hepatocarcinogenesis initiated with NDEA and promoted by carbon tetrachloride. An understanding of how cancer may be prevented is one of the key objectives of the recent researches. This can be achieved to some extent by using chemopreventive agents, naturally occurring or synthetic, that can suppress or prevent the processes of tumor development. Therefore, it is essential to identify agents as well as to evaluate their efficacy and to elucidate their mechanisms of action. In the present study, serum obtained from tumor bearing rats showed significant increase in AST, ALT and ALP activities along with significant increase in total bilirubin compared to control animals. The elevation of these enzyme activities was indicative of the toxic effect of NDEA on the liver tissue associated with sever histological distortions (figure 2b-d). It is known that N-nitroso compounds act as strong carcinogens in various mammals including primates [29]. NDEA has been shown to be metabolized by cytochrome P-450 IIE1 (CYP 2E1) to its active ethyl radical metabolite, which could interact with DNA causing mutation and carcinogenesis [30]. Administration of prepared new thiopyrimidine acyclic nucleosides and thioglycoside analogs including 2, 3b, 3c, 4a and 4c to NDEA treated rats showed restoration of AST, ALT and ALP activities and total bilirubin level towards normal especially in group treated with 3b and 3c. Such reverse in serum enzyme activities could be attributed to the ability of these compounds to inhibit CYP 2E1 expression (as shown in figure 3), presumably by serving as a competitive inhibitor, leading to a decrease in the formation and/or bioactivation of these nitrosamines. The improvement in the biochemical parameters was accompanied with improvement in the histopathological abnormalities especially in 3b and 3c (Figure, 2f and g) which showing hepatocytes maintaining near-normal architecture while the other compounds revealed moderate improvement of hepatic histopathology over NDEA group. Tyrosine kinases play a critical role in the modulation of growth factor signaling. Activated forms of these enzymes can cause increases in tumor cell proliferation and growth, induce antiapoptotic effects, and promote angiogenesis and metastasis. In addition to activation by growth factors, protein kinase activation by somatic mutation is a common mechanism of tumorigenesis [31]. In consistent with the above fact, our results showed that highly significant increase in the expression of TRK in NDEA-treated group, while the administration of prepared compounds (2, 3b, 3c, 4a and 4c) in the NDEA-treated rats resulted in significantly inhibitory potential against TRK for all the tested compounds comparing with the NDEA group. Compounds 3b and 3c were the most potent inhibitor against TRK expression.

Tumor cell transformation is a multistage process. An *In Situ* tumor after a period of time abruptly sparks the formation of new blood vessels from the preexisting vasculature a process termed as angiogenesis or neovascularization. Tyrosine kinase plays an important role in this process [32]. This process though occurs normally during embryonic development, female reproductive cycle or wound healing is found as a crucial step in tumor transition from benign to malignant form, capable of spreading throughout the body [33]. Antiangiogenic drugs stops new vessels from forming around a tumor and break up the existing network of abnormal capillaries that feeds the cancerous mass, thus shrinks the tumor by limiting blood supply [34]. To study the anti-angiogenesis effect of the prepared compounds we measured the level of VEGF and our results showed that there was over production of VEGF after administration NDEA. This is in concurrently with Torimura *et al.*[35] who stated that VEGF was over expressed intoxicated with NDEA. Inhibition of tumor growth by neutralization of VEGF has been verified by treatment with the prepared compounds which showed decreased amount of VEGF in the cancer-bearing animals, thereby inhibiting the formation of new blood vessel and tumor growth in the order of 3c > 3b > 4a > 2> 4c.

It has been proposed that sialic acid appears to be highly sensitive marker for the progression of tumor growth and its angiogenesis [36]. Previously, Rachesky *et al.* [37] have been reported an increased level of glycoproteins in animals exposed to carcinogen NDEA. In the present study, serum total sialic acid level was estimated and found to be significantly elevated ($p<0.05$) in NDEA-treated rats (figure 6). The exact cause in rising of sialic acid levels in tumorigenesis is not known, however, various theories are attributed to such increment as: alterations in the cell surface during cell transformation, stimulation of the liver by tumor growth to synthesize glycoproteins or increased glycosylation [38]. Also, neoplastic transformation could lead to sialic acid elevation through the shedding of sialic acid from the tumor cell surface or possibly as a product of the tumor itself [39]. Administration of prepared compounds leads to significant decrease in TSA level especially in 3b and 3c groups. Consequently, this suggests that these

compounds played an important role against NDEA-induced hepatocarcinogenesis by maintaining TSA status.

In the present study, results showed that the activities of heparanase and elastase enzymes were very highly significantly increased in NDEA-treated group as compared with control. The treatment with prepared compounds (2, 3b, 3c, 4a and 4c) resulted in decrease in the activity of both enzymes especially 3b and 3c (Figure, 7). In the meantime, several studies suggested that targeting the activity of heparanase and elastase might be a beneficial antitumor therapy for liver cancer [40]. Few studies on the potency of heparanase as a marker for HCC were found in the literature [41]. Heparanase is a heparan sulfate (HS) degrading endoglycosidase participating in extracellular matrix degradation and remodeling. Apart of its well-characterized enzymatic activity, heparanase was noted to exert also enzymatic-independent functions, which include enhanced adhesion of tumor-derived cells and primary T-cells [42]. Heparanase seems to modulate two critical systems involved in tumor progression, namely vascular epidermal growth factor (VEGF) expression and epidermal growth factor receptor (EGFR) activation. Neutralizing heparanase enzymatic and non-enzymatic functions is therefore expected to profoundly affect tumor growth, angiogenesis and metastasis [43]. A large number of publications clearly link heparanase expression to the process of tumorigenesis in a wide number of cancers, which were reviewed by Zhang *et al.* [44]. Collectively, they suggest that heparanase plays a fundamental role in sustaining the pathology of malignant diseases and therefore it may provide a potential target for anti-cancer therapy [45].

Elastase is another broad-range proteolytic enzyme thought to be a tumor promoter involved in increasing tumor cell invasiveness by facilitating cell motility and transendothelial migration as it has the ability to degrade basement membrane and ECM glycoproteins such as elastin, fibronectin, as well as adhesive molecules and junctional cadherins [46]. Moreover, elastase considered to be the only protease that is able to degrade insoluble elastin, a structural component of elastic tissues such as blood vessel, skin, lung, liver and breast tissues [47]. Furthermore, Taniguchi *et al.*[48] postulated that increased elastase destroy the barrier between tumor and the local circulatory system, either lymphatic or hematogenous, and result in at least loco-regional metastases.

In conclusion, the results of our study clearly indicate a beneficial effect of prepared new thiopyrimidine acyclic nucleosides and thioglycoside analogs including 2, 3b, 3c, 4a, and 4c on chemically-induced rat liver tumorigenesis. To our knowledge, this is the first experimental evidence of the chemopreventive activity of thiopyrimidine acyclic nucleosides and thioglycoside analogs. Under our experimental conditions, the tested compounds especially 3b and 3c exert their antitumor activity through affecting the process of angiogenesis and metastasis and may be potent anticancer agents for inclusion in modern clinical trials after more investigations on higher animals.

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