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Artesunate obliterates experimental hepatocellular carcinoma in rats through suppression of IL-6-JAK-STAT signalling

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ABSTRACT

Activation of the IL-6 mediated JAK-STAT (Janus associated kinase-signal transducer and activator of transcription) oncogenic signalling plays a major role in hepatocellular carcinoma pathogenesis. The aim of this study is to assess the anti-tumour, anti-proliferative and apoptotic potential of artesunate and its capacity to modulate JAK-STAT pathway in a nitrosodiethylamine mediated experimental hepatocellular carcinoma model. Administration of nitrosodiethylamine (200 mg/kg body weight by i.p. Injections) to rats resulted in alterations of liver pathophysiological parameters such as increased relative liver weight, and increased tumour nodule occurrence. It also increased the levels of serum marker enzymes (AST, ALT, ALP, LDH, and γGT) and tumour biomarker (AFP) levels suggestive of its capacity to cause liver tumourigenesis. Additionally, the immunohistochemistry of liver sections pertaining to nitrosodiethylamine administered animals showed increased detection of AgNOR, PCNA, and GST-Pi positive cells suggestive of its capacity to promote liver proliferation associated tumourigenesis. On the contrary, artesunate (25 mg/kg bodyweight) supplementation to nitrosodiethylamine administered animals decreased all the above mentioned pathophysiological, biochemical, and immunohistochemistry parameters suggesting its anti-tumour and anti-proliferative potential. Furthermore, immunoblot analysis showed significant up-regulation of IL-6, GP130, JAK-2, STAT-3 (pY705), Bcl-xL, Bcl-2 and simultaneous down-regulation of Caspase-3, PARP and SOCS-3 in nitrosodiethylamine administered animals. Nevertheless, the immunoblot analysis revealed vice-versa on artesunate supplementation to nitrosodiethylamine administered animals, indicating promotion of the feedback loop inhibition mechanism through SOCS3 up-regulation thereby leading to suppression of JAK-STAT signalling. Overall all these findings substantiate that artesunate promotes anti-tumour, anti-proliferation and apoptosis against nitrosodiethylamine mediated hepatocellular carcinoma.

1. Introduction

Hepatocellular carcinoma (HCC), remains a major health issue and ranks third leading cause of cancer related mortality worldwide [1–3]. The chief risk factors for HCC include hepatitis B and C viral infections, excessive alcohol consumption, hemochromatosis, obesity and exposure to environmental carcinogens, such as aflatoxins. The surgical curative options presently available restrict to resection and transplantation nevertheless they have been ineffective in advanced stages of HCC. Nonsurgical chemotherapy (Sorafenib or Sunitinib) based procedures, however, have resulted in undesirable side effects. Both the surgical and nonsurgical clinical modalities presently are effective only in less than 10% of HCC patients. The advanced treatment strategy currently available is highly expensive and henceforth lacks wide accessibility to the poor. Present prognosis with best available treatments has not increased the overall survival rate by more than two years in 60% of patients [4]. Therefore, there is an urgent need to develop cheap, safe and effective therapy against HCC.

One such plant derived compound Artesunate (ATS), a semisynthetic derivative of Artemisinin isolated from *Artemisia annua* L., is an anti-malarial agent with potent anti-cancer effects.

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Numerous studies suggest ATS possess biological and improved pharmacological activities, including anti-viral, anti-inflammatory, anti-tumour, anti-angiogenic, and anticancer effects [5–11]. Myriad groups independently have reported that the ATS inhibits inflammation and prevents chemo-resistance in many cancer cell lines *in vitro* and *in vivo*, such as breast, lung, colon, liver, and gastric cancer [10,12–14]. Nevertheless, there is very little available information *in vivo* about the anti-tumour, anti-proliferative or apoptosis favouring effects of ATS and its mechanism of action against hepatocellular carcinoma [7,10,11].

Our preliminary findings suggest that ATS (IC_{50}—20 μM) inhibited constitutive and IL-6-induced activation of signal transducers and activators of transcription 3 (STAT-3) *in vitro* in HepG2, HuH-7 and PLC/PRF5 cells in a dose and time dependent manner [15]. Recently, a cohort based clinical finding concluded that IL-6 promotes HCC only through the activation of STAT-3 and not through any other STAT members [16]. Further apprehension on the biological association of other STAT members in relation to HCC progression by various other cytokines is still unclear and beyond the scope of this study. Henceforth, this study limits to understanding the potential for development of ATS as a novel HCC therapeutic agent based on mechanistic regulation of IL-6 mediated JAK-STAT signalling cascade in nitrosodiethylamine (NDEA) induced liver tumour model. NDEA is a widely accepted, potent, liver specific carcinogen that closely mimics a subclass of human HCC [17,18]. Various studies on NDEA mediated HCC and cell-targeted perturbations of JAK-STAT activity have confirmed the role of IL-6 in driving the "proliferation and tumour" axis in HCC [18,19]. Hence the present study is vital to analyse and provide new insights in decoding the molecular and therapeutic aspects of ATS against NDEA-mediated IL-6/JAK/STAT signalling to affirm its role in obliteration of HCC.

2. Materials and methods

2.1. Experimental animals

Male Wistar rats weighing between 250 and 350 g (12 weeks old) were procured from the Central Animal Facility, SASTRA University and the whole study was performed here in controlled conditions of temperature 24 ± 2 °C, relative humidity 50–56% and photo schedule 12 h:12 h of light:dark. Animals were fed with a standard pellet diet (Amrut Feeds, Mumbai, India) and water *ad libitum*. The experiments were carried out in strict accordance with the guidelines set by CPCSEA (Committee for the purpose of control and supervision of experiments on animals), India and the experimental design were approved by the institutional animal ethics committee (IAEC approval No.: 197/SASTRA/IAEC/RPP).

2.2. Experimental design

We grouped the animals into four experimental groups, each comprising six animals analyzed for a total experimentation period of 16 weeks. The grouping was as follows (Fig. 1): Group 1 was the control rats fed with standard pellet diet and pure drinking water; Group 2 was the diseased group, for which NDEA (200 mg/kg body weight) was injected intraperitoneally, once a week for 6 weeks. Group 3 rats were induced with NDEA (200 mg/kg body weight) for 6 weeks, subsequently treated with ATS (25 mg/kg body weight) orally, twice a week from 7th experimental week. Group 4 was drug control group and ATS (25 mg/kg body weight) alone was supplemented to the rats, through oral gavage from week 7 till the end. After 16 weeks, all the rats were anesthetized after overnight fasting and then euthanized. Serum was separated from the blood and used for the analysis of biochemical parameters. We immediately excised the liver tissue, rinsed in ice-cold saline and stored the frozen tissue for further histological and molecular level investigations.

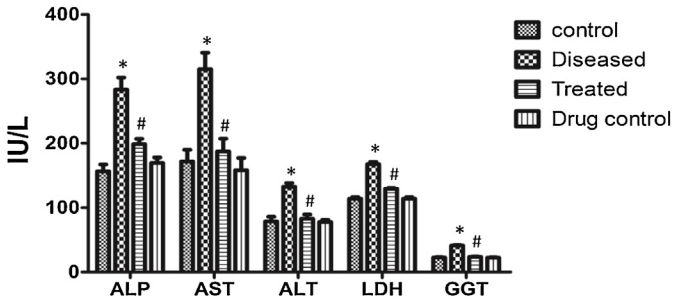

Fig. 1. Effect of ATS on serum liver marker enzymes: alkaline phosphatase (ALP), aspartate transferase (AST), alanine transferase (ALT), lactate dehydrogenase (LDH) and gamma-gluthathione transferase (γ-GT) in control and experimental animals. Results are expressed as mean ± SD (N = 6). $p < 0.05$ was considered significant: * for diseased vs. control, and # for diseased vs. treated (One way ANOVA, GraphPad Prism).

2.3. Analysis of alpha fetoprotein (AFP) level

The tumour marker α-fetoprotein was measured in blood serum using solid phase enzyme linked immunosorbent assay (ELISA) [20]. Briefly, 20 μL of standards and samples were added to the appropriate wells. 100 μL of buffer was added to each well and mixed for 10s. Wells were covered and incubated at room temperature for 30 min. After aspirating the contents, the wells were washed five times. Enzyme conjugate (150 μL) was added to each well and mixed for 5 s. After incubation for 30 min, TMB (3, 30, 5, 50-tetramethylbenzidine) substrate solution (200 μL) was added to all the wells and the plate was incubated in the dark at room temperature. Enzyme activity was stopped by the addition of 2M H_2SO_4 (50 μL). After gentle mixing, when the blue colour is completely changed to yellow, absorbance was measured at 450 nm. The absorbance was measured for each set of reference standards and serum samples. The amount of AFP (ng/mL) in each sample was quantified against the standard curve obtained by plotting the absorbance of the reference standards against its concentration. Values were expressed as ng/mL.

2.4. Biochemical assays

Activities of aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH) and gamma-glutamyltranspeptidase (γ-GT) were assayed in the serum [21–23].

2.5. Liver histopathology

Histological evaluation was carried out in the caudal liver and it was fixed in formalin solution and embedded in paraffin wax. Sections were sliced at 4 μm thickness, and stained with hematoxylin and eosin (H&E) stains. Histological changes were viewed and recorded under light microscope [24]. The number and size of tumour nodules formed were measured in each group to denote the severity of histopathological changes.

2.6. Argyrophilic nucleolar organizer region staining

Argyrophilic nucleolar organizer region (AgNOR) staining was done as previously reported [25]. Briefly, gelatin (2 g/L) was dissolved in aqueous formic acid (1 mL/L). To this solution, 1:2 v/v of aqueous silver nitrate solution (50 g/L) was added. The paraffin embedded tissue slides were deparaffinised in xylene, hydrated