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# Novel Biochemical Pathways for 5-Fluorouracil in Managing Experimental Hepatocellular Carcinoma in Rats

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**Abstract** Five fluorouracil (5-FU) is extensively used in the treatment of hepatocellular carcinoma (HCC). It is well documented that 5-FU and its metabolites inhibit DNA synthesis through inhibition of thymidylate synthetase. Little is known about additional pathways for 5-FU in managing HCC. The present experiment was mainly designed to study possible biochemical pathways that can be added to 5-FU's mechanisms of action. Four groups of rats constituted a control group (given saline only), a trichloroacetic acid group (TCA, 0.5 g/kg/day for 5 days, orally), a 5-FU-positive group (75 mg/kg body weight, intraperitoneally, once weekly for 3 weeks) and a TCA-treated with 5-FU group (24 h from last TCA dose). We executed both biochemical—serum alpha-fetoprotein (AFP), liver tissue contents of total glycosaminoglycan (TGAGs), collagen (represented as hydroxyproline), total sialic acid (TSA), free glucosamine (FGA) and proteolytic enzyme activity (as pepsin and free cathepsin-D—and histological examinations of the liver tissue. The results revealed histological changes such as central vein congestion and irregularly shaped, substantially enlarged, vesiculated and binucleated hepatocytes. The nuclei were mostly polymorphic and hyperchromatic, and several vacuolation was noticed in the cytoplasm encircling the nucleus with masses of acidophilic material. 5-FU greatly corrected these changes, except that some necrotic and cytotoxic effects of 5-FU were still shown. AFP was significantly elevated in TCA-intoxicated, but reversed in 5-FU-treated, groups. Increased proteolytic activity by TCA was reversed by 5-FU, which also restored TGAG contents to normal; but both TCA and 5-FU depleted collagen content. TCA significantly elevated FGA but depressed TSA; this action was reversed by 5-FU treatment. In conclusion, it is possible that proteolytic activity, expressed as upregulated pepsin and free cathepsin-D activities, is increased in HCC. This is accompanied by extracellular matrix macromolecular disturbance, manifested as decreased TGAGs, collagen and TSA, with marked increase in FGA liver tissue content. The elevated FGA with depressed TSA content of liver tissue may be attributed to a cancer-hampered *N*-acetylation of FGA into SA. 5-FU administration markedly depressed hepatic tissue proteolysis, possibly reactivated *N*-acetylation of FGA into SA and elevated TGAGs without stopping tissue fibrosis as collagen was not affected. This study explores additional pathways for the mechanism of action of 5-FU, through conservation of extracellular matrix composition in situ, inhibiting invasion and metastasis in addition to its DNA-disturbing pathway.

**Keywords** Hepatocellular carcinoma · Cathepsin-D · Pepsin · Glycosaminoglycan · Sialic acid · Collagen · Glucosamine · 5-Fluorouracil · Liver

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## Introduction

5-Fluorouracil (5-FU) belongs to fluoropyrimidine family that shows wide application in the management of hepatocellular carcinoma (HCC). It is characterized by a high

hepatic extraction rate with a relatively low hepatosystemic toxicity (Milano and Chamorey 2002). It is widely assumed that both cytotoxic drugs and ionizing radiation kill tumor tissue through initiation of apoptosis (Dimanche-Boitrel et al. 2005). Most of the cytotoxic mechanisms of 5-FU are well outlined. Fluorouracil inhibits cell proliferation by (1) forming fluorodeoxyuridine monophosphate, which in turn blocks thymidylate synthase, the enzyme that catalyzes de novo synthesis of the DNA precursor thymidylate; (2) forming defective F-RNA, which ultimately interferes with protein synthesis; and (3) forming defective, fluorinated DNA, which results in single-strand breaks and DNA fragmentation (Sobrero et al. 1997, 2000; Kinsella et al. 1997). Of these mechanisms, thymidylate synthase inhibition is generally considered the most important for continuous-infusion regimens, whereas F-RNA formation may dominate in high-dose bolus treatments (Sobrero et al. 1997). 5-FU is toxic only when taken up by cells and anabolized to fluoronucleotides, which in turn may be incorporated into nucleic acids or bind to thymidylate synthase. The bioavailability of 5-FU is greatly limited by rapid catabolism in the blood, liver and other organs. After i.v. injection in humans, the drug has a half-life in blood of only 8–20 min (Heggie et al. 1987). 5-FU and its metabolite 5-fluoro-2-deoxyuridine mainly inhibit thymidylate synthetase, so they block DNA synthesis (Ishikawa 2008). In 1995, it was reported that carcinoma tissue shows disturbed mucopolysaccharide and collagen metabolism due to possible proteolytic enzyme perturbations (Bhuvarahamurthy and Govindasamy 1995). Proteinases and growth factors are involved in remodeling of HCC (Schoedel et al. 2003). HCC is the third most frequent cause of cancer death worldwide (Tabor 2001). Animal and human studies have shown that genotoxic and cytotoxic effects and inflammatory responses caused by HCC inducers, like viral infections with B or C, chronic ethanol abuse, dietary exposure to aflatoxin $B_1$ or other genotoxic compounds such as nitrosamines from diet or tobacco smoke, are key events in entraining hepatocytes to hepatocarcinogenesis (Zhu and Fung 2000; Ohshima et al. 2003). Extracellular matrix (ECM) constitutes a three-dimensional supporting scaffold in which tumor cells proliferate, invade and influence differentiation and morphogenesis. It consists mainly of collagen, elastin, glycosaminoglycans (GAGs) and glycoproteins (Bernfield and Banerjee 1972). Tissue collagen is frequently evaluated as representative hydroxyproline (HP) content. Thus, HP is considered as an important index in hepatic tissue changes in response to different pathologic insults (Garcia et al. 2002). Lysosomes possess many hydrolytic enzymes which play an important role in the local degradation of both collagen and proteoglycans. This step is critical in the malignant progression of tumors (Kojima et al. 1975). The incidence of HCC is one million new cases per year (El-Serag and Mason 1999). Treatment of HCC relies on three main options: hepatic resection, percutaneous destruction of the tumor (by radiofrequency) and orthotopic liver transplantation (Bruix and Sherman 2005). In the present study, the biochemical role of 5-FU in managing HCC induced by trichloroacetic acid (TCA) is outlined. This was achieved by determining proteolytic activity, represented by liver tissue pepsin and free cathepsin-D activities and ECM components, including GAGs, HP, free glucosamine (FGA) and total sialic acid (TSA) contents. Both liver histology and serum alpha-fetoprotein (AFP) were taken as markers for cancer initiation and response to anticancer treatment. The first mentioned parameters were pursued to find out new biochemical pathways for the cytotoxic potential of 5-FU on HCC.

## Materials and Methods

### Materials

#### *Chemicals*

All chemicals were of analytical grade and came from local suppliers. 5-FU was purchased as 250 mg/ml ampoules (Pharco, Alexandria, Egypt).

#### *Experimental Design*

Thirty-two male Wistar rats weighing 190 ± 10 g were randomly assigned for the study purpose. The animals were housed in polyethylene cages in a moderately humid room under a controlled 12 h light and 12 h dark cycle and fed ordinary rodent diet and water ad libitum for 1 week before dosing. They were classified into four groups, each consisting of eight rats. The first group served as control, in which animals were given 0.5 ml physiological saline i.p. daily for 1 month. The second group served as the liver cancer group, given a daily oral dose of TCA (Sigma-Aldrich, St. Louis, MO), for five consecutive days, by gavage as 500 mg/kg body weight, previously dissolved in distilled water, neutralized by NaOH to a pH of 5–7.5 (Tao et al. 2000). Animals of the third group were given 5-FU i.p. as 75 mg/kg body weight, diluted with saline, once per week for three consecutive weeks (Winocur et al. 2006). Animals of the fourth group were given TCA for 5 days and, 24 h later, 5-FU was given for 3 weeks. The mortality rate in the TCA group was around 20%. All animals were killed 1 day after a total treatment interval of 30 days (i.e., 4 days after the last 5-FU dose); livers were excised, washed with saline and divided into two portions—one

kept in 10% formol saline (for histological examination) and one frozen directly in liquid nitrogen and kept at −80°C. Serum samples were also kept frozen on the day of analytical investigations.

## Methods

### *Determination of Serum AFP Levels*

We determined, using ELISA UBI AFP quantitative CM-101 kits (MagiWells, Ladera Ranch, CA) following the instructions of the manufacturer (Belanger et al. 1973). For determination of liver tissue collagen, HP was primarily extracted from tissue by acidic hydrolysis in 6 N HCl and subjected to heating in a water bath for 3 h at 130°C in sealed tubes (Woessner 1961); then, HP content was colorimetrically estimated in the hydrolysate (Neuman and Logan 1950) against standard HP (Sigma).

### *Determination of TGAGs*

A given liver tissue weight was extracted by five volumes of 4 M guanidinium HCl (UFC, India) in 0.05 M sodium acetate, containing 0.01 M disodium EDTA and 0.005 M benzamidine HCl (Merck, Darmstadt, Germany) (both used as protease inhibitors) (Oegema et al. 1975); then, TGAGs were colorimetrically estimated in the hydrolysate using a solution of alcian blue 8GX (BDH, Poole, UK) against a standard heparin sodium (Nile Co., Cairo, Egypt) (Gold 1979; Folch et al. 1957). In the defatted tissue, FGA was determined against standard glucosamine HCl (BDH) (Elson and Morgan 1933) as modified by Pari and Saravanan (2006).

### *Determination of TSA*

In the previously defatted tissue hydrolysate, TSA content was estimated according to the method of Warren (1959), against standard SA (Sigma). Prior to determination of both pepsin and free cathepsin-D activities, substrate (1% egg albumin) total protein was determined by the method of Lowry et al. (1951); it was 670 mg/dl. Tissue was extracted with ice-cold water (Kumar et al. 2000). In this aqueous extract, pepsin activity was monitored by the method of Acharya and Katyare (2004). Free cathepsin-D activity was determined in acidified protein substrate according to the method of Nerurkar et al. (1988). Enzyme activity in both cases was expressed as micrograms of released tyrosine per milligram substrate protein. Colorimetric estimation of tyrosine, as the product of proteolysis, was conducted by diluted Folin–Ciocalteu (FC) reagent (Folin and Ciocalteau 1927) against tyrosine standard (Kumar et al. 2000; Acharya and Katyare 2004). Tissue changes were investigated by light microscopy after hematoxylin–eosin staining.

### Statistical Analysis

The data were expressed as means ± SE. Statistical significance was examined by one-way analysis of variance (ANOVA) (Duncan 1955). $P < 0.05$ was assumed to be statistically significant.

## Results

Administration of TCA significantly elevated AFP levels, an action greatly reversed by 5-FU administration. Pepsin activity was nonsignificantly upregulated by TCA administration; however, 5-FU alone or after TCA treatment significantly downregulated pepsin activity. Free cathepsin-D activity was significantly upregulated by TCA, but 5-FU either alone or given after TCA nonsignificantly restored this activity to less than the normal value. HP content was significantly depressed by all treatment methods. TGAGs were nonsignificantly depressed by TCA but nonsignificantly elevated by 5-FU and significantly elevated in TCA-intoxicated animals when treated with 5-FU. FGA content was significantly increased after HCC induction; 5-FU significantly reversed this action either alone or after TCA intoxication. TSA content was significantly decreased after HCC induction but restored to near normal by 5-FU either alone or after induction (Table 1). Histological examination of liver tissue in control animals revealed normal parenchymal cells with granulated cytoplasm and small, uniform nuclei, radially arranged around the central vein, as well as normal sinusoids and normal architecture (Fig. 1a). TCA-treated rats showed loss of normal architecture with oval or irregularly shaped hepatocytes, central vein congestion and many transformed liver cells of foci that were substantially enlarged, largely vesiculated and frequently binucleated, which were clearly distinguishable from the surrounding normal parenchyma. The nuclei were mostly found to be polymorphic and hyperchromatic; several nuclei were large and hyperchromatic (basophilic) with prominent and centrally located nucleoli. Additionally, extensive vacuolation was noticed in the cytoplasm encircling the nucleus with masses of acidophilic (eosinophilic) material. The sinuses were greatly dilated (Fig. 1b). 5-FU-treated rats showed normal central vein, normal architecture and a normal pattern of liver cell plates with dilated sinusoids; some liver cells around the central vein showed ballooning, which may have been due to a cytotoxic effect of 5-FU (Fig. 1c). Liver sections of TCA- and 5-FU-treated rats showing reversible changes still present as central vein congestion and

**Table 1** Variations in serum AFP level (ng/ml); hepatic activity of pepsin and free cathepsin-D; and liver tissue content of HP, TGAGs, FGA and TSA after oral administration of TCA (0.5 g/kg/day for 5 days) and i.p. treatment with 5-FU (75 mg/kg/week/3 weeks)

| Variable | Group | | | |
|---|---|---|---|---|
| | Control | TCA | 5-FU | TCA + 5-FU |
| AFP (ng/ml) | 7.0 ± 1.63 | 270.5 ± 17* | 7.5 ± 1.5 | 118.4 ± 4.6* |
| Pepsin activity (U/l) | 15.5 ± 0.13 | 15.8 ± 0.13 | 13.2 ± 0.4* | 14 ± 0.43* |
| Free cathepsin-D activity (U/l) | 5.1 ± 0.11 | 6.2 ± 0.17* | 5 ± 0.11 | 5 ± 0.15 |
| HP (μg/g wet tissue) | 5.19 ± 0.17 | 1.58 ± 0.1* | 1.1 ± 0.06* | 1.8 ± 0.1* |
| TGAGs (μg/g wet tissue) | 45.5 ± 1.9 | 44.4 ± 1.27 | 47.6 ± 0.8 | 52.6 ± 1.9* |
| FGA (μg/g wet tissue) | 58.29 ± 1.23 | 141.04 ± 6* | 19.9 ± 0.95** | 15.9 ± 0.98* |
| TSA (μg/g wet tissue) | 44.1 ± 1.4 | 8.6 ± 0.6* | 39.4 ± 0.83 | 44.5 ± 3.07 |

Values are expressed as mean ± SE ($n = 8$)

* Significantly different from control at $P < 0.01$

ballooning but generally less than in the cancer (TCA-treated) group indicated some improvement. Some cells were necrotic, possibly due to the cytotoxic effect of 5-FU on hyperchromatic cells, as shown in Fig. 1d.

## Discussion

Tumorigenesis is a multistep process in which a series of genetic alterations result in an imbalance in cell division and cell death (Hanahan and Weinberg 2000). Our results showed that administration of TCA significantly elevated AFP levels. This action supports the hepatocarcinogenicity of TCA as an experimental model (Lumpkin et al. 2003). Histological findings showed that TCA induced dysplastic hepatocellular changes, periportal congestion, fatty change, multinucleolosis and increased nuclear size, all considered to be early signs of HCC supported by an increase in AFP. A single oral TCA dose was previously reported to induce peroxisomal proliferation and genotoxicity in rats (Parnell et al. 1986). These results were in agreement with a recently reported observation about the hepatocarcinogenic action of this dose in rats (Abdel-Hamid 2009). Much work on connective tissue biochemistry and cell biology has led to a greater understanding of biochemical mechanisms that contribute to tumor invasion and metastasis. Alterations of GAGs in animal tumors (Asokan et al. 1989) and in human hepatic tumors (Kojima et al. 1975) have been reported. In the present work, TGAGs were nonsignificantly downregulated in hepatic tissue by TCA intoxication. This action was significantly reversed by 5-FU chemotherapy. It was early reported that the transition from in situ to invasive carcinoma is always accompanied by marked disorganization and localized loss of basement membrane components such as collagen and laminin (Bhuvarahamurthy and Govindasamy 1995; Liotta et al. 1986). TCA administration significantly depressed tissue collagen synthesis. Also, 5-FU treatment depressed collagen synthesis, whether given alone or after TCA treatment. The downregulatory action of 5-FU on collagen synthesis is possibly attributed to blockade of transforming growth factors necessary for collagen synthesis (Wendling et al. 2003). However, 5-FU efficacy in preventing invasion and metastasis may be due to its maintenance of GAG content in tissues (Bhuvarahamurthy and Govindasamy 1995). In the present work, TCA elevated the activity of pepsin (nonsignificantly) and free cathepsin-D (significantly). 5-FU administration significantly decreased pepsin activity, whether used alone or after TCA intoxication. It also nonsignificantly reduced cathepsin-D activity by the same two dosing protocols.

Seemingly, TCA induced activation of both pepsin and cathepsin-D activities, possibly taking part in TGAG and collagen degradation in the ECM (Nakajima et al. 1987). The increased hepatic lysosomal enzyme activity, represented as elevated cathepsin-D activity, was primarily considered a tumor initiator in chemically induced HCC (Lundholm et al. 1980; Premalatha and Sachdanandam 2000).

Generally, 5-FU cytotoxicity was referred to changes in gene expression of cell cycle-controlling factors. Thus, overexpression of p53 target genes involved in the cell cycle and apoptosis (*CDKN1A/p21*, *TP53INP*, *TNFRSF6/FAS* and *BBC3/PUMA*); significant repression of *Myc*, in addition to a higher regulation of the mitochondrial death genes *APAF1*, *BAK1* and *BCL2*; and induction of genes of the ID family are major pathways for 5-FU antitumor action. Also, a direct causal relationship between *p21*, *ID1* and *ID2* overexpression increasing acetylation of histones H3 and H4 and binding of p53 to their promoters as a result of 5-FU treatment has been reported (Hernandez-Vargas et al. 2006).

Modulation of proteolytic activity after 5-FU treatment may be considered an additional pathway in liver cancer management. This may be supported by the hypothesis that assumes an increased proteolytic activity in hepatic metastasis (Woolley 1984). In our study, FGA tissue

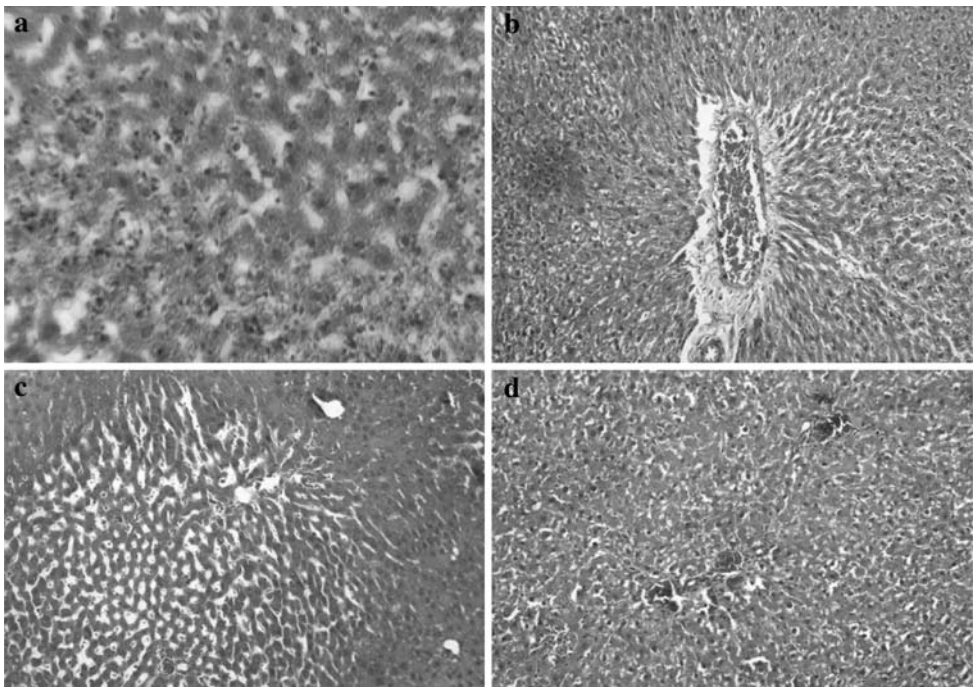


**Fig. 1** **a** Photomicrograph of liver section in normal control rat (H&E, 400×). **b** Photomicrograph of liver section in TCA-treated rat (H&E, 400×). **c** Photomicrograph of liver section in 5-FU-treated rat (H&E, 400×). **d** Photomicrograph of liver section in TCA + 5-FU-treated rat (H&E, 400×)

content was significantly upregulated by TCA intoxication and significantly depressed beyond the normal value by 5-FU if given alone or after TCA intoxication. The use of different GAG components in liver cancer diagnosis and prognosis provides a reliable tool in the management of HCC. This is mostly due to the fact that many glycoproteins are produced by the liver (Isailovic et al. 2008). On the other hand, glucosamine constitutes the principal component of SA. It is also the precursor of all hexoamines that form SAs and proteoglycans (Deal and Moskowitz 1999). The present results demonstrate that TSA tissue content was significantly downregulated by TCA intoxication. 5-FU treatment, whether alone or after TCA-induced HCC, restored TSA content to near normal.

Reportedly, liver tissue glycoproteins are released rapidly into the circulation. These macromolecules could be used as seromarkers for liver tissue damage (Akamatsu et al. 1976). The significantly decreased TSA content in our study may induce an increase in serum levels, which may help as a seromarker in HCC diagnosis and follow-up (Kongtawelert et al. 2003).

In conclusion, it is possible that proteolytic activity, expressed as upregulated pepsin and free cathepsin-D activities, is increased in HCC. This is accompanied by ECM macromolecular disturbance, manifested as decreased TGAGs, collagen and TSA, with a marked increase in FGA liver tissue content. The elevated FGA with depressed TSA content of liver tissue may be attributed to a cancer-induced inhibition of *N*-acetylation of FGA into SA. 5-FU administration markedly depressed hepatic tissue proteolysis, possibly reactivated *N*-acetylation of FGA into SA and elevated TGAGs without stopping tissue fibrosis as collagen was not affected.

This study adds novel pathways for the chemotherapeutic potential of 5-FU, through modulation of ECM protein reorganization.

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