JBUON 2014; 19(4): 1048-1054
ISSN: 1107-0625, online ISSN: 2241-6293 • www.jbuon.com
E-mail: editorial_office@jbuon.com

ORIGINAL ARTICLE

Cytotoxic effects of suramin against HepG2 cells through activation of intrinsic apoptotic pathway

Ahmed Tayel[1], Mohamed A Ebrahim[2], Ahmed S Ibrahim[1], Amal M El-Gayar[1], Mohammed M Al-Gayyar[1,3]

[1]Department of Clinical Biochemistry, Faculty of Pharmacy, University of Mansoura, Mansoura, Egypt; [2]Oncology Center, University of Mansoura, Mansoura, Egypt; [3]Faculty of Pharmacy, University of Tabuk, Tabuk, Saudi Arabia

Summary

Purpose: *To investigate the cytotoxic activity of suramin against HepG2 human HCC cell line.*

Methods: *HepG2 cells were treated with 15, 30 and 45 μM suramin. HepG2 cell proliferation was measured by MTT and lactate dehydrogenase (LDH) assays. Heparan sulfate proteoglycans (HSPGs), glucuronic acid and glucosamine levels were measured. Moreover, apoptosis was assessed by measuring caspase-8 and caspase-9 activities. The effect of suramin on HepG2 cells was compared with the same doses of a standard drug, cisplatin.*

Results: *Suramin blocked heparanase leading to dose-dependent increase in HSPGs and reduction in glucuronic acid and glucosamine levels. Suramin reduced HepG2 cells survival and showed cell cytotoxicity in a dose-dependent manner with LD50 45.04 μM, compared with cisplatin (LD50 28.9 μM) ($p<0.05$). Moreover, suramin was able to increase the activity of caspase-9 but not of caspase-8.*

Conclusion: *Suramin possesses cytotoxic properties, which can be partially explained by its ability to inhibit heparanase and restore HSPGs. Suramin activates intrinsic apoptosis without affecting the extrinsic pathway.*

Key words: *caspase-8, caspase-9, glucosamine, glucuronic acid, heparanase, heparan sulfate proteoglycans*

Introduction

Hepatocellular carcinoma (HCC) is one of the most common solid malignancies. It has become the third most common malignancy worldwide with very poor prognosis, rendering it the fourth highest cause of cancer-related deaths [1]. In Africa, liver cancer has been ranked as the fourth most common cancer, and most of liver cancers are HCC [2]. Moreover, HCC ranks second in incidence and mortality among men in Egypt [3].

Recent studies [3-6] suggest that sulfated polysaccharides could affect tumor cells directly. Sulfated polysaccharides could inhibit proliferation and metastasis of tumor cells. Some sulfated polysaccharides can induce apoptosis and differentiation of tumor cells, but the mechanism is uncertain [7].

Heparanase is a mammalian endoglycosidase that degrades heparan sulfate at the cell surface and in the extracellular matrix of a wide range of tissues [8]. The expression of heparanase was detected in a wide variety of human malignant tumors and was closely associated with tumor invasion, metastasis and angiogenesis [9]. Heparanase cleavage of heparan sulfate (HS) in the extracellular matrix, particularly in epithelial and subendothelial basement membranes, leads to disassembly of extracellular barriers, release of HS-bound angiogenic factors and/or growth factors and generation of bioactive HS fragments that promote growth factor–receptor binding, dimerization, and signaling [10-12]. However, heparanase is involved in fundamental biological phenomena associated with cancer progression,

Correspondence to: Mohammed M Al-Gayyar, PhD. Faculty of Pharmacy, University of Tabuk, Tabuk 71491,Saudi Arabia. Tel: +966 5 08114998, Fax: +966 144237748, E-mail: mhgayyar@yahoo.com
Received: 15/04/2014; Accepted: 30/04/2014

including cell survival, invasion, proliferation, neovascularization, and creation of a growth-permissive microenvironment [13,14].

Suramin is a polysulphonated naphthyl urea, which has been used successfully for sleeping sickness, onchocerciasis and some infections caused by parasites. In animals and patients, high doses of suramin dramatically increase tissue glycosaminoglycans through elevations in the concentration of circulating HS, due, in part, to inhibition of heparanase enzymes responsible for HS intracellular degradation. Therefore, we conducted the following study to evaluate the cytotoxic activity of suramin in an *in vitro* model of HCC through its effect on HSPGs and apoptosis.

Methods

Drugs

Cisplatin vials (10mg/10ml) were obtained from Merck Co. (White Rouse Station, NJ, USA). Suramin was obtained from Tocirs Co. (Bristol, UK).

Cell line

In all experiments the HepG2 cell line was used. HepG2 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% streptomycin and penicillin, and incubated for 24 hrs at 37°C in a 5% CO_2 incubator to allow them to grow.

Cell lysates

HepG2 cells were extracted using a lysis buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% Triton X-100, supplemented with a cocktail of protease inhibitors (Roche, South San Francisco, CA, USA). Protein concentration was determined using Bradford dye (BioRad, Hercules, CA, USA). In this colorimetric assay for measuring total protein concentration (Bradford dye-binding method), the color of the dye Coomassie Brilliant Blue changes from brown to blue in response to various protein concentrations.

MTT assay

1x104 cells were plated in each well of 96-well plates, and placed in humidified 5% CO_2 incubator at 37°C to allow them to grow for a 24-h period. Cells were exposed to different concentrations of suramin and ciplatin (15, 30 and 45 μM) and placed in humidified 5% CO_2 incubator for 48 hrs. Cells incubated in culture medium alone served as a control for cell viability (untreated wells). Cell viability was determined using the MTT assay as described previously by our group [5].

Cytotoxicity with LDH

The supernatant was collected from each well and centrifuged for 5 min at 700 g to remove cell debris. The supernatant was placed in a second 96-well plate. LDH solution was added to each well including controls and cell-free wells. The plate was allowed to develop for 20 min in the dark at room temperature. Cytotoxicity with LDH was determined by subtracting the normalized absorbance at 680 nm of the cell-free wells from the normalized absorbance of wells with cells. Relative cytotoxicity was determined by normalizing against the positive cytotoxicity control, 1% Triton X-100.

Assessment of oxidative stress

This was estimated in the cell lysates through the following parameters:

1. H_2O_2 concentration was measured by modified horseradish peroxidase (HRPO) method after modification as described previously by Shams et al. [6]. This method depends on the HRPO mediated oxidation of phenol red by released H_2O_2, which resulted in the formation of a compound that could be read at 610 nm.
2. Superoxide anion concentration was measured by nitroblue tetrazolium (NBT) method [15]. It depends on the ability of the superoxide anion to reduce NBT to an insoluble formazan that could be measured at 560 nm.
3. Superoxide dismutase (SOD) activity was determined using the phenazine methosulfate method [16], in which the ability of the enzyme to inhibit the phenazine methosulfate-mediated reaction of nitrobluetetrazolium dye is measured as indication of the activity of SOD.
4. Reduced glutathione (GSH) concentration was measured [17] based on the reduction of 5,5'-Dithiobis (2-nitrobenzoic acid) (DTNB) glutathione, forming a highly colored yellow anion that could be measured at 412 nm.

Determination of degradation products of heparan sulfate

Glucosamine was measured using the dimethylaminobenzaldehyde method [18] and glucuronic acid was measured using the naphthoresorcinol method [19].

Enzyme-linked immunosorbent assay (ELISA)

HSPGs and heparanase were measured using commercially available ELISA kit (USCN Life Science Inc. Houston, TX, USA; and Biotang Inc., Waltham, MA, USA, respectively) according to the manufacturer's instructions.

Estimation of caspase-8 and -9 activities

Caspase-8 and -9 enzyme activities were measured colorimetrically using commercially available kits (GenScript, Piscataway, NJ, USA) according to the manufacturer's instructions.

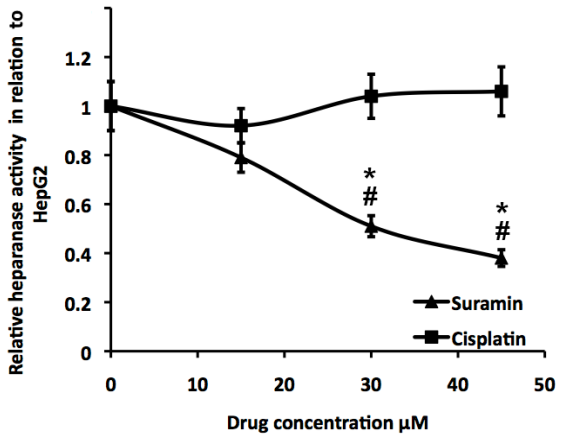

Figure 1. Effects of suramin and cisplatin (15,30 and 45μM) on heparanase activity in HepG2 cells. * significant as compared with HepG2 cells without treatment at p<0.05. # significant as compared with HepG2 cells treated with the corresponding concentration of cisplatin at p<0.05..

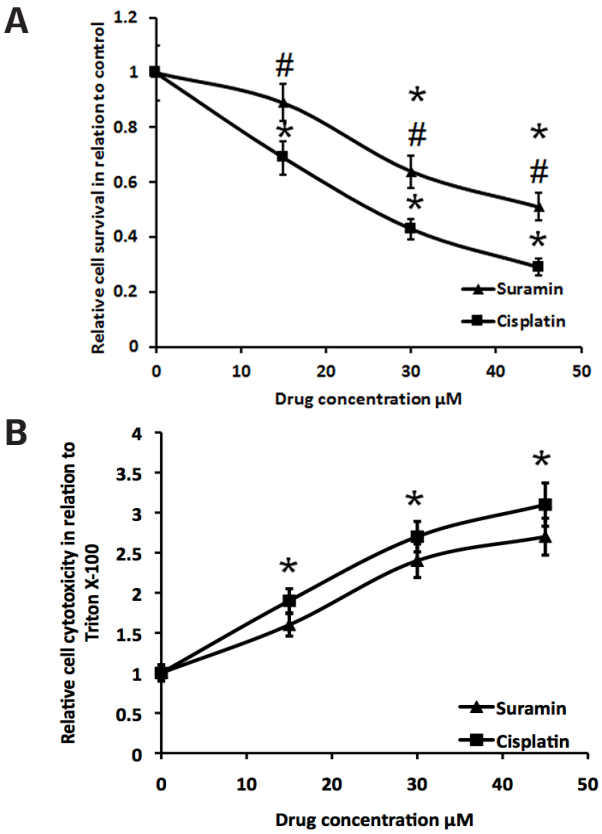

Figure 2. Effects of suramin and cisplatin (15,30 and 45μM) on HepG2 cell survival **(A)** and cell cytotoxicity **(B)**. * significant as compared with HepG2 cells without treatment at p<0.05. # significant as compared with HepG2 cells treated with the corresponding concentration of cisplatin at p<0.05.

Statistics

For descriptive statistics of quantitative variables, mean±standard error (SE) were used. Normality of the sample distribution of each continuous variable was tested with the Kolmogorov–Smirnov (K–S) test. One-way analysis of variance (ANOVA) was used to compare means between groups. If differences existed among the means, *post hoc* Bonferroni correction test was used. Statistical computations were done on a personal computer using Microsoft Excel 2007 and statistical significance was set at p < 0.05.

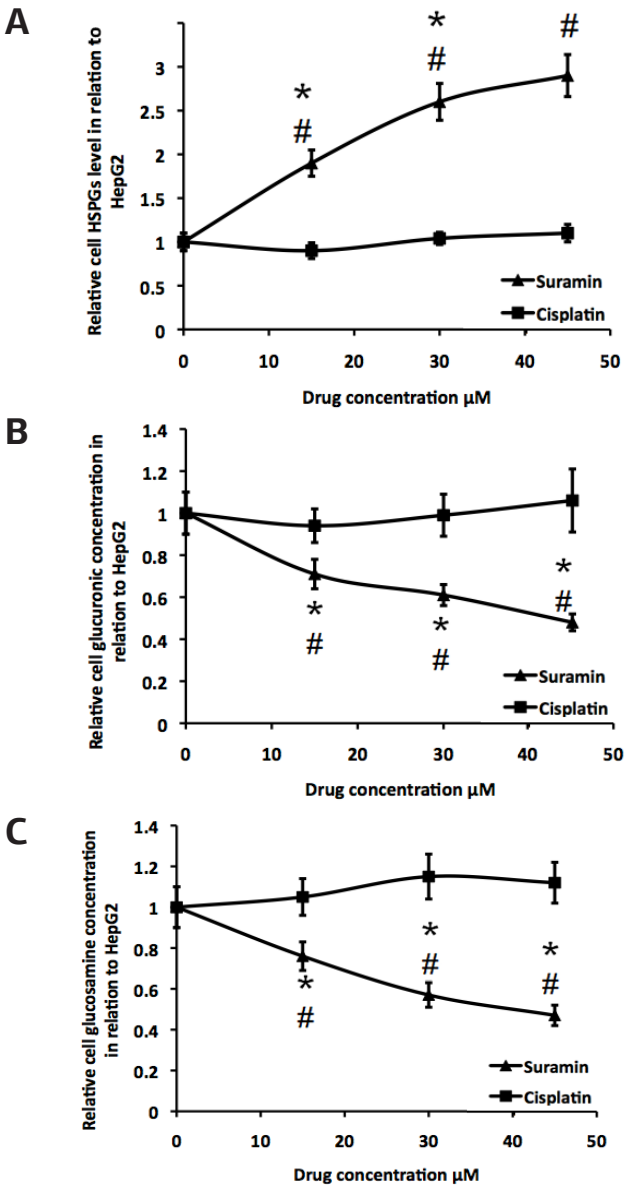

Figure 3. Effects of suramin and cisplatin (15,30 and 45μM) on the concentration of HSPGs **(A)**, glucuronic acid **(B)** and glucosamine **(C)** in HepG2 cells. * significant as compared with HepG2 cells without treatment at p<0.05. # significant as compared with HepG2 cells treated with the corresponding concentration of cisplatin at p<0.05.

Results

Suramin blocked HCC-induced elevation of heparanase in HepG2 cells

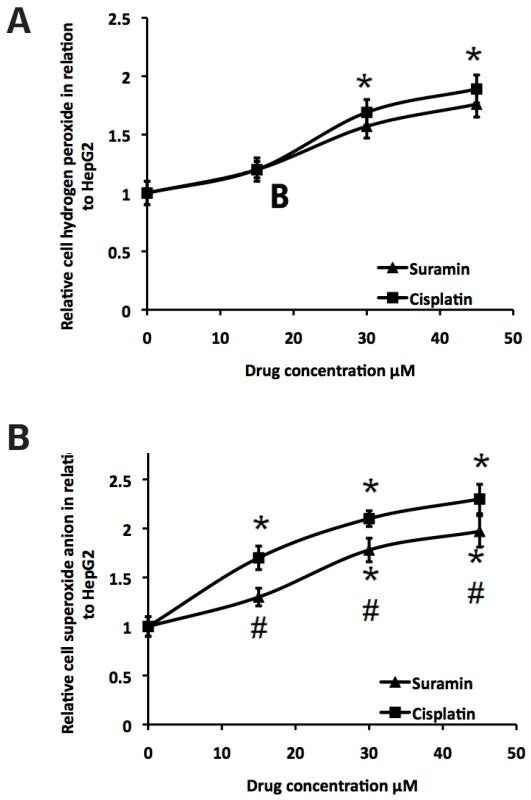

Figure 4. Effects of suramin and cisplatin (15,30 and 45µM) on the concentration of hydrogen peroxide **(A)** and superoxide anion **(B)** in HepG2 cells. * significant as compared with HepG2 cells without treatment at p<0.05. # significant as compared with HepG2 cells treated with the corresponding concentration of cisplatin at p<0.05.

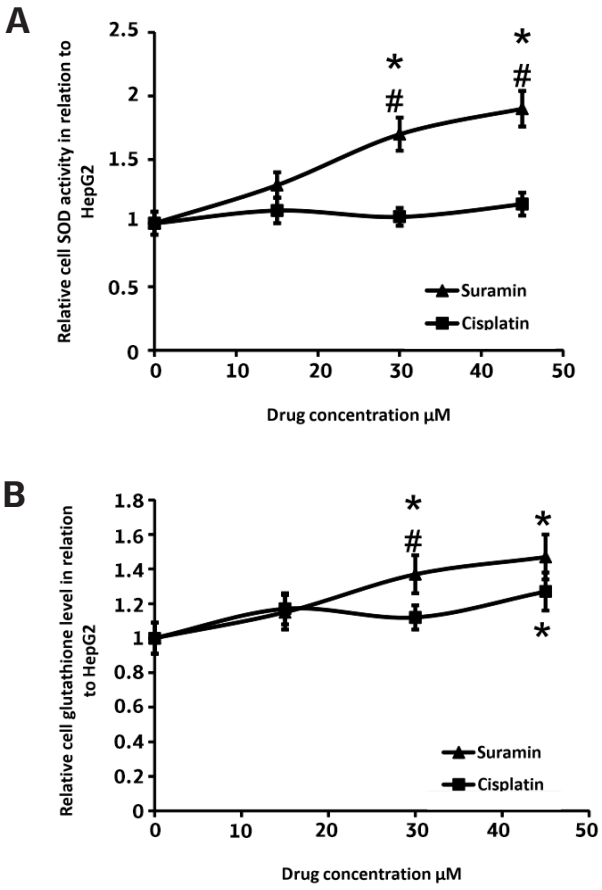

Figure 5. Effects of suramin and cisplatin (15,30 and 45µM) on the activity of SOD **(A)** and concentration of reduced glutathione **(B)** in HepG2 cells. * significant as compared with HepG2 cells without treatment at p<0.05. # significant as compared with HepG2 cells treated with the corresponding concentration of cisplatin at p<0.05

Treatment of HepG2 cells with suramin showed more than 60% reduction in heparanase activity (Figure 1). Treatment of HepG2 cells with cisplatin did not affect heparanase activity.

Cytotoxic effect of suramin in HepG2 cells

Next, we evaluated the cytotoxic effects of suramin on HepG2 cells and found that treatment of cells with suramin showed dose-dependent reduction in the cells'viability (Figure 2a) and enhanced cytotoxicity (Figure 2b). Cisplatin (LD_{50} 28.9 µM) produced significantly higher cytotoxic effects on HepG2 cells viability as compared with suramin (LD_{50} 45.04 µM; p<0.05).

Effect of suramin against HSPGs and its degradation products in HepG2 cells

HepG2 cells treated with suramin showed significant dose-dependent increase in HSPGs as well as dose-dependent reduction in glucuronic acid and glucosamine as compared with HepG2 cells without treatment. Treatment of HepG2 cells with cisplatin did not affect the concentration of HSPGs (Figure 3).

Effects of suramin against HCC-induced oxidative stress in HepG2 cells

We found that suramin resulted in dose-dependent elevation in superoxide anion, H2O2, SOD and reduction of glutathione. In parallel, treatment of HepG2 cell with cisplatin significantly increased superoxide anion and H_2O_2 levels without affecting the levels of SOD and reduced glutathione (Figures 4 and 5).

Effect of suramin on apoptosis in HepG2 cells

Treatment of HepG2 cells with suramin produced dose-dependent increase in the activities of caspase-9, while it pro-

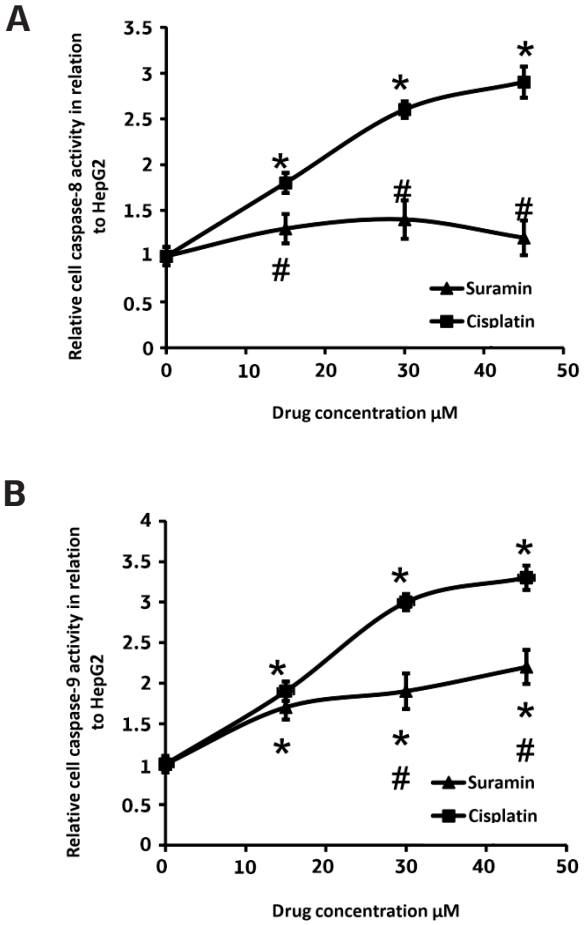

Figure 6. Effects of suramin and cisplatin (15,30 and 45µM) on the activity of caspase-8 **(A)** and caspase-9 **(B)** in HepG2 cells. * significant as compared with HepG2 cells without treatment at p<0.05. # significant as compared with HepG2 cells treated with the corresponding concentration of cisplatin at p<0.05.

duced no significant effect on the activity caspase-8 as compared with HepG2 cells without treatment (Figure 6). Moreover, cisplatin produced significantly higher effects on the activity of both caspases in HepG2 cells as compared with suramin.

Discussion

HCC is one of the most frequently diagnosed cancers worldwide. The disease is predominant in Africa, but its incidence is steadily increasing throughout the rest of the world. Most HCCs develop in patients with a history of chronic hepatitis or cirrhosis in which there is continuous inflammation and regeneration of hepatocytes [5]. Due to limited treatment options for advanced HCC, HCC has a poor prognosis.

Heparanase is the only endo-β-D-glucuronidase that can cleave HSPGs. Heparanase is an ideal target for anticancer immunotherapy against HCC because it is specifically overexpressed in HCC [20]. Heparanase expression is increased in many kinds of tumors and this elevation is often associated with more aggressive disease and poor prognosis due to advanced local and distant metastases [21]. Heparanase, produced by malignant tumor cells, can mediate the degradation of HSPGs in the basement membrane and extracellular matrix, which act as hurdles for metastasis by limiting the invasion and spread of tumor cells [22]. Treatment with heparanase inhibitors markedly reduced the incidence of metastasis in experimental animals [23,24]. Identification of inhibitors of heparanase is an attractive approach towards developing new therapeutics for metastatic tumors. We previously reported the in vivo antitumor activity of suramin, a polysulphonated naphthyl urea, against HCC [4]. We found that treatment of HepG2 cells with suramin significantly reduced heparanase activity as well as significantly increased HSPGs concentration. Suramin was reported to silence heparanase [25,26]. In addition, suramin produced cytotoxic effects on HepG2 and reduced their viability. The cytotoxic activity of suramin is superior to that of cisplatin on HepG2 cells.

Depending on the origin of death stimuli, there are two major mechanisms of apoptosis called the intrinsic or mitochondrial, and the extrinsic or death receptor, pathways. The intrinsic pathway involves mitochondrial outer-membrane permeability leading to release of apoptogenic factors such as cytochrome C and apoptosis-inducing factor. The final stage involves the activation of caspase-9 and subsequently caspase-3 activation [27,28]. Once released to cytosol, cytochrome C, together with pro-caspase-9, form an apoptosome, which in turn activates the cleavage of caspase-9 and the downstream effector caspase-3 [29-31]. However, we found that suramin results in a dose-dependent increase in caspase-9 in HepG2 cells that was associated with significant increase in oxidative stress markers.

The extrinsic pathway is induced by specific ligands such as TNF-α and TNF-related apoptosis-inducing ligand (TRAIL) receptors. These ligands lead to the recruitment and activation of initiator cysteine aspartic proteases (caspases) such as caspases-8 and 10 [27]. In turn, caspase-8 can propagate the apoptosis signal by direct cleavage of caspase-3 [32]. The active caspase-3 involves DNA fragmentation, nuclear fragmentation, membrane blebbing and other morphological and

biochemical changes [33]. We found that suramin did not affect caspase-8 activity in HepG2 cells.

Conclusions

The main findings of the current study are that suramin possesses cytotoxic properties, which can be partially explained by its ability to: (1) inhibit heparanase and restoration of HSPGs; (2) generation of oxidative stress; and (3) activation of the intrinsic apoptosis pathway. However, suramin has no effects on the extrinsic pathway.

References

1. Liu S, Li Y, Chen W et al. Silencing glypican-3 expression induces apoptosis in human hepatocellular carcinoma cells. Biochem Biophys Res Commun 2012; 419: 656-661.
2. Soliman AS, Hung CW, Tsodikov A et al. Epidemiologic risk factors of hepatocellular carcinoma in a rural region of Egypt. Hepatol Int 2010;4:681-690.
3. Metwaly HA, Al-Gayyar MM, Eletreby S, Ebrahim MA, El-Shishtawy MM. Relevance of serum levels of interleukin-6 and syndecan-1 in patients with hepatocellular carcinoma. Sci Pharm 2012;80:179-188.
4. Tayel A, Abd El Galil KH, Ebrahim MA, Ibrahim AS, El-Gayar AM, Al-Gayyar MM. Suramin inhibits hepatic tissue damage in hepatocellular carcinoma through deactivation of heparanase enzyme. Eur J Pharmacol 2014;728:151-160.
5. Darweish MM, Abbas A, Ebrahim MA, Al-Gayyar MM. Chemopreventive and hepatoprotective effects of Epigallocatechin-gallate against hepatocellular carcinoma: role of heparan sulfate proteoglycans pathway. J Pharm Pharmacol 2014;66:1031-1045.
6. Shams M, Al-Gayyar M, Barakat E, Ebrahim M, El-Shishtawy M. Circulating adiponectin: a potential prognostic marker for hepatocellular carcinoma. Chin Ger J Clin Oncol 2011;10:570-574.
7. Wu XZ, Chen D. Effects of sulfated polysaccharides on tumour biology. West Indian Med J 2006;55:270-273.
8. Iozzo RV. Heparan sulfate proteoglycans: intricate molecules with intriguing functions. J Clin Invest 2001;108:165-167.
9. Chen Z, Zhu L, Li X et al. Down-regulation of heparanase leads to the inhibition of invasion and proliferation of A549 cells in vitro and in vivo. Acta Biochim Biophys Sin (Shanghai) 2013;45:188-193.
10. Vlodavsky I, Elkin M, Abboud-Jarrous G et al. Heparanase: one molecule with multiple functions in cancer progression. Connect Tissue Res 2008;49:207-210.
11. Elkin M, Ilan N, Ishai-Michaeli R et al. Heparanase as mediator of angiogenesis: mode of action. FASEB J 2001;15:1661-1663.
12. Kato M, Wang H, Kainulainen V et al. Physiological degradation converts the soluble syndecan-1 ectodomain from an inhibitor to a potent activator of FGF-2. Nat Med 1998;4:691-697.
13. Edovitsky E, Elkin M, Zcharia E, Peretz T, Vlodavsky I. Heparanase gene silencing, tumor invasiveness, angiogenesis, and metastasis. J Natl Cancer Inst 2004;96:1219-1230.
14. Vlodavsky I, Friedmann Y, Elkin M et al. Mammalian heparanase: gene cloning, expression and function in tumor progression and metastasis. Nat Med 1999;5:793-802.
15. Baehner RL, Boxer LA, Davis J. The biochemical basis of nitroblue tetrazolium reduction in normal human and chronic granulomatous disease polymorphonuclear leukocytes. Blood 1976;48:309-313.
16. DeChatelet LR, McCall CE, McPhail LC, Johnston RB Jr. Superoxide dismutase activity in leukocytes. J Clin Invest 1974;53:1197-1201.
17. Beutler E, Duron O, Kelly BM. Improved method for the determination of blood glutathione. J Lab Clin Med 1963;61:882-888.
18. Elson LA, Morgan WT. A colorimetric method for the determination of glucosamine and chondrosamine. Biochem J 1933;27:1824-1828.
19. Mosher RE. Interferences in the colorimetric determination of glucuronic acid in blood serum. Clin Chem 1962;8:378-388.
20. Han XM, Wang HJ, Yang JM et al. Effects of anti-heparanase antibody on the growth and invasion of HC-CLM6 human hepatocellular carcinoma cells]. Zhonghua Zhong Liu Za Zhi 2009;31:10-14.
21. Arvatz G, Shafat I, Levy-Adam F, Ilan N, Vlodavsky I. The heparanase system and tumor metastasis: is heparanase the seed and soil? Cancer Metastasis Rev 2011;30:253-268.
22. Xiong Z, Lu MH, Fan YH et al. Downregulation of heparanase by RNA interference inhibits invasion and tumorigenesis of hepatocellular cancer cells in vitro and in vivo. Int J Oncol 2012;40:1601-1609.
23. Cassinelli G, Lanzi C, Tortoreto M et al. Antitumor efficacy of the heparanase inhibitor SST0001 alone and in combination with antiangiogenic agents in the treatment of human pediatric sarcoma models. Biochem Pharmacol 2013;85:1424-1432.
24. Dredge K, Hammond E, Handley P et al. PG545, a dual heparanase and angiogenesis inhibitor, induces potent anti-tumour and anti-metastatic efficacy in preclinical models. Br J Cancer 2011;104:635-642.
25. Ho M. Advances in liver cancer antibody therapies: a focus on glypican-3 and mesothelin. BioDrugs 2011;25:275-284
26. Marchetti D, Reiland J, Erwin B, Roy M. Inhibi-

tion of heparanase activity and heparanase-induced angiogenesis by suramin analogues. Int J Cancer 2003;104:167-174.

27. Fulda S, Debatin KM. Extrinsic versus intrinsic apoptosis pathways in anticancer chemotherapy. Oncogene 2006;25:4798-4811.
28. Bayomi HS, Elsherbiny NM, El-Gayar AM, Al-Gayyar MM. Evaluation of renal protective effects of inhibiting TGF-beta type I receptor in a cisplatin-induced nephrotoxicity model. Eur Cytokine Netw 2013;24:139-147.
29. Reed JC. Mechanisms of apoptosis. Am J Pathol 2000; 157:1415-1430.
30. Gao F, Fu Z, Tian H, He Z. The Euphorbia lunulata Bge extract inhibits proliferation of human hepatoma HepG2 cells and induces apoptosis. J BUON 2013;18:491-495.
31. Guo Y, Zhang W, Yan YY et al. Triterpenoid pristimerin induced HepG2 cells apoptosis through ROS-mediated mitochondrial dysfunction. J BUON 2013;18:477-485.
32. Schattenberg JM, Nagel M, Kim YO et al. Increased hepatic fibrosis and JNK2-dependent liver injury in mice exhibiting hepatocyte-specific deletion of cFLIP. Am J Physiol Gastrointest Liver Physiol 2012;303: G498-506.
33. Portt L, Norman G, Clapp C, Greenwood M, Greenwood MT. Anti-apoptosis and cell survival: a review. Biochim Biophys Acta 2011;1813:238-259.