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

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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,
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% CO₂ 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% CO₂ 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% CO₂ 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. H₂O₂ 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 H₂O₂, 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 nitroblue tetrazolium 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.
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.

**Results**

*Suramin blocked HCC-induced elevation of heparanase in HepG2 cells*
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 (LD₅₀ 28.9 µM) produced significantly higher cytotoxic effects on HepG2 cells viability as compared with suramin (LD₅₀ 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 H₂O₂ 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-
Reduced no significant effect on the activity caspase-8 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 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.

**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 polysulfonated 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


