CYTOTOXICITY OF SORAFENIB IN HUMAN HEPATOCELLULAR CARCINOMA CELLS

BY

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ABSTRACT

Hepatocellular carcinoma (HCC) is the predominant type of primary liver malignancy with high rates of mortality worldwide. Most HCC tumors are inherently resistant to chemotherapy and despite the tremendous advances in cancer chemotherapy, their treatment remains quite challenging.

Sorafenib, a multikinase inhibitor, has recently been approved for the treatment of advanced HCC. The present study aimed to further explore the potential cytotoxic activities of sorafenib in HepG2 cells as well as the possible underlying mechanisms. Thus, HepG2 cells were treated with different concentrations of sorafenib. The concentration that inhibited the growth of the cells by 50% was calculated from the fitted survival curves. The effect of sorafenib on cell cycle, apoptosis and proliferation was investigated. Sorafenib- induced cytotoxicity in HepG2 cells. This could be partially attributed to increased apoptosis by augmenting the level of active caspase-3. Moreover, sorafenib induced cell cycle arrest and had anti-proliferative effects by decreasing the level of p-Akt.

Keywords: Sorafenib; Hepatocellular carcinoma; Apopotosis; Cell cycle arrest.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the second leading cause of cancer death worldwide **(Jemal et al., 2011)** and is highly resistant to systemic chemotherapy **(Zhu, 2010)**. Hepatocellular carcinoma usually occurs in the developing countries of Asia and Africa where most of the new cases occur **(El-Serag and Rudolph, 2007)**.

Sorafenib, an orally active multikinase inhibitor, is currently the first and only molecular target drug clinically approved for the treatment of advanced HCC **(Sun et al., 2014)**. Sorafenib inhibits both cell surface tyrosine kinase receptors and downstream intracellular serine/threonine kinases in the Ras (rat sarcoma viral oncogenes)/mitogen activated protein kinase (MAPK) cascade **(Adnane et al., 2006)**. Receptor tyrosine kinases inhibited by sorafenib include vascular endothelial growth factor receptor (VEGFR) -1, -2 and -3, platlet derived growth factor receptor (PDGFR) -β, c-KIT, FMS-like tyrosine kinase 3 (FLT-3) and RET **(Wilhelm et al., 2006)**. Sorafenib is generally regarded as an anti-angiogenic agent although the exact mechanism of its action is not fully understood **(Chan and Yeo, 2014)**. The aim of the present study was to further investigate the potential cytotoxic activities exerted by sorafenib in HepG2 cells as well as the possible underlying mechanisms particularly its effects on apoptosis, cell cycle and cell proliferation.

EXPERIMENTAL

Materials

Sorafenib was purchased from LC Laboratories (Woburn, MA, USA). It was dissolved in dimethyl sulfoxide (DMSO) and stored in aliquots at -20˚C. Chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) and cell culture materials were purchased from Gibco Life Technologies Ltd. (Grand Island, NY, USA).

Cells line

HepG2 cells were purchased from the National Cancer Institute, Cairo, Egypt. The cells were kept in RPMI-1640 medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin antibiotic. The cells were maintained in a humidified 5% (v/v) CO_2 atmosphere at 37˚C.

Assessment of protein content

The method of Bradford **(Bradford, 1976)** was used to determine the protein content in cell lysate and cellular extract.

Cytotoxicity assay

HepG2 cells were seeded at a density of 5000 cells/well in 96-well flat bottom plates and left to attach for 24 h. Then, the cells were treated with a serial concentration of sorafenib. The control wells were treated with 0.3% DMSO, the vehicle, only. After 72 h, the cytotoxicity was assessed using Sulphorhodamine-B (SRB) method as previously reported **(Skehan et al., 1990)**. The absorbance of the wells were measured at 545 nm with an ELISA microplate reader (ChroMate-4300, FL, USA). From the fitted survival curves, the drug concentrations that inhibited the growth of the cells by 50% (IC_{50}), were calculated (Graph Pad, Prism software, version 5). This concentration, 2.3 µM, was used for further mechanistic studies and all treatments were carried out for 72 h.

Flowcytometric cell cycle analysis

HepG2 cells were plated in T_{75} flasks at a density of 1×10^6 cells/flask in RPMI-1640 supplemented medium and treated with the indicated drug concentration. Then, cells were trypsinized and washed twice with phosphate buffer saline (PBS). The CycleTEST^{TM} PLUS DNA Reagent Kit (BD Biosciences, San Jose, CA) was used to stain the cellular DNA according to manufacturer`s protocol. Cell cycle analysis was performed using the Becton-Dickinson FACScan flow cytometer (BD Biosciences, San Jose, CA) and the CELLQuest software (BD Biosciences, San Jose, CA).

Determination of the level of active caspase-3

The active caspase-3 ELISA kit (R&D Systems, Minneapolis, MN) was used to determine the level of active caspase-3. HepG2 cells were seeded in 6-well plates at a density of 5×10^4 cells/well and treated with the appropriate drug concentration. Cells were incubated for 72 h. The cellular extracts were prepared according to the manufacturer`s instructions and the level of active caspase-3 was determined according to the kit`s protocol.

Assessment of p-Akt level

The RayBio® phosphor-Akt (Ser473) ELISA kit (RayBiotech, Inc., Norcross, GA, USA) was used to determine the level of p-Akt (Ser473). The procedure was carried out according to the manufacturer`s protocol.

Statistical analysis

Data were presented as mean ± SD. Individual groups were compared using the unpaired Student's t-test. Multiple comparisons were performed using one way analysis of variance (ANOVA) followed by Dunnett test for post hoc analysis. The level of significance was set at $p < 0.05$. All analyses were performed using GraphPad InStat software, version 3.05 (GraphPad Software, La Jolla, CA). Graphs were sketched using GraphPad Prism software, version 5.00 (GraphPad Software, La Jolla, CA).

RESULTS

Sorafenib induced cytotoxicity in HepG2 cells

Treatment of HepG2 cells with different concentrations of sorafenib (0.3125-20 μM) for 72 h significantly decreased the growth of cells in a concentration-dependent manner. From the fitted survival curves, the IC_{50} of sorafenib was calculated and was found to be 2.3 μM. (Fig.1).

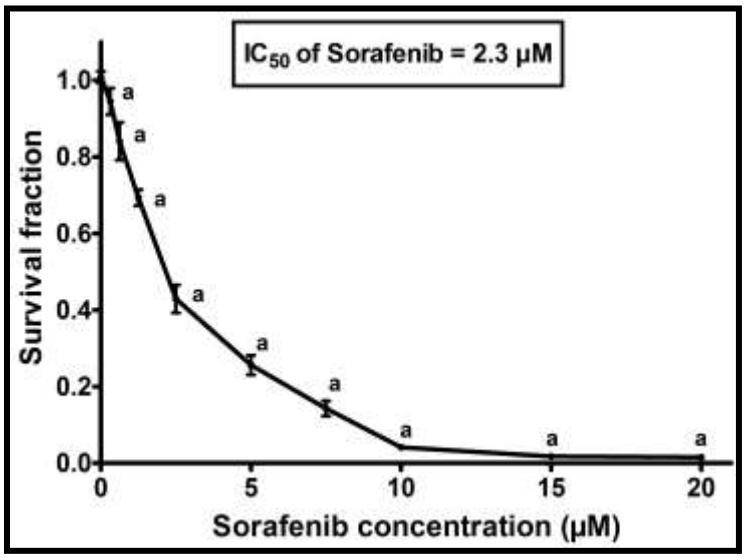

Fig. 1. Cytotoxicity of various concentrations of sorafenib in HepG2 cell line. [a]p <0.05: Statistically significant when compared to the control value using ANOVA followed by Dunnett test as post-hoc test.

Sorafenib induced cell cycle arrest

Cell cycle distribution was evaluated using flowcytometric analysis. Treatment of HepG2 cells with sorafenib caused a significant cell cycle arrest at the G_0/G_1 phase on the expense of S and G_2/M phases. Moreover, sorafenib induced a significant increase in the percentage of apoptotic cells. (Table 1).

Table 1: Effects of sorafenib on cell cycle distribution in HepG2 cells

Groups*	G_0/G_1	S	G_2/M	Apoptotic cells
Control	60.77±1.92	29.62±3.75	7.94±2.79	1.67±0.09
Sorafenib	68.77±0.57[a]	24.21±1.2	4.27±0.55	2.75±0.16[a]

*Values are presented as percentage of cells at the indicated cell cycle phases ± SD. a: Significantly different from the control group, P < 0.05 using unpaired Student's t-test.

Sorafenib significantly enhanced the apoptosis of HepG2 cells

To investigate the effect of sorafenib on the apoptotic machinery, the level of active caspase-3 was measured. Fig. 2 shows that active caspase-3 level was increased significantly following treatment with sorafenib by 217% as compared to the control values.

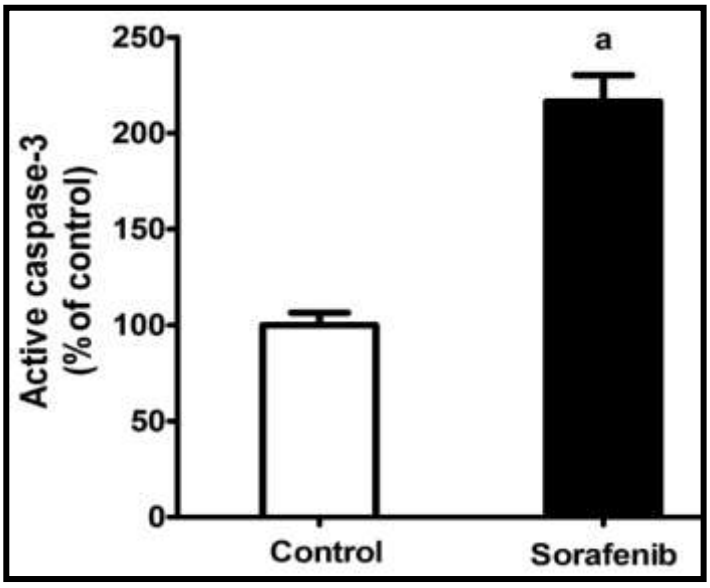

Fig. 2. Effect of sorafenib on active caspase-3 level. Data is given as percentage of control.

Each point is the mean ± SD. The experiment was done in triplicates. a: Significantly different from the control, $P < 0.05$ using unpaired Student's t-test.

Sorafenib significantly downregulated the level of p-Akt

To study the effects of sorafenib on cell proliferation the level of p-Akt was assessed. Treatment of HepG2 cells with sorafenib significantly decreased the level of p-Akt by 65.87% as compared to the control values. (Fig. 3).

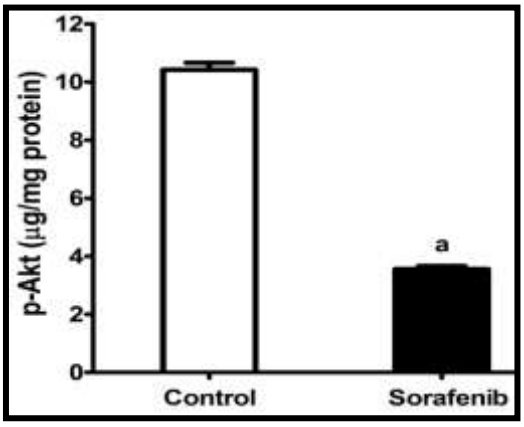

Fig. 3. Effect of sorafenib on p-Akt level. Each point is the mean ± SD. The experiment was done in triplicates. a: Significantly different from the control, $P < 0.05$ using unpaired Student's t-test.

DISCUSSION

Hepatocellular carcinoma tumors are inherently resistant to chemotherapy **(Huang and Liu, 1999)**. Additionally, conducting of controlled clinical trials in HCC patients has been hindered by the aggressive nature of the disease and the occurrence of HCC in the developing world where access to clinical trials may be difficult **(Zhu, 2006)**. Recently, targeted therapy have achieved higher specificity towards cancer cells, thus limiting nonspecific toxicities. Tyrosine kinases represent an especially important target due to their role in the modulation of growth factor signaling **(Arora and Scholar, 2005)**.

In 2008, sorafenib, a multikinase inhibitor, was approved for the treatment of advanced HCC on basis of the results of two randomized, double-blind, placebo-controlled, multicenter phase III trials: the Sorafenib Hepatocellular Carcinoma Assessment Randomized Protocol (SHARP) trial **(Llovet et al., 2008)** and a trial conducted in patients from the Asia-Pacific region **(Cheng et al., 2009)**. Although sorafenib is generally regarded as an inhibitor of angiogenesis, its exact mode of action is not fully understood **(Chan and Yeo, 2014)**. Thus, the aim of the present study was to explore the potential cytotoxic activities of sorafenib in the HCC cell line, HepG2, as well as the putative underlying mechanisms. Moreover, the effects of sorafenib on apoptosis, cell cycle and proliferation were investigated. Initially sorafenib showed cytotoxicity in HepG2 cells where the IC_{50} was found to be 2.3 μM. Previously, sorafenib proved to be cytotoxic in HCC cell lines **(liu et al., 2006)**.

Cell cycle analysis using flowcytometry was carried out to determine the distribution of cells in the cell cycle. Sorafenib caused a preferential cell cycle arrest at the G_0/G_1 phase. The ability of sorafenib to induce cell cycle arrest in G_0/G_1 phase was previously reported **(Tao et al., 2014; Kong et al., 2014)**. However, it should be mentioned that sorafenib has been reported to induce also the accumulation of cells at the $S/G_2/M$ phases **(Fernando et al., 2012)**. This may indicate that sorafenib abilities to induce cell cycle arrest varies in different cell lines. The increased percentage of apoptotic cells required further investigation of the apoptotic machinery.

Accordingly, the level of active caspase-3 was assessed. Indeed, sorafenib significantly elevated caspase-3 level in accordance with the results obtained in the cell

cycle analysis. To investigate the anti-proliferative effects of sorafenib, the level of p-Akt was measured. Sorafenib is essentially an inhibitor of the Raf/MEK/ERK signaling pathway in HepG2 cells **(Liu et al., 2006)**. This study further demonstrate that sorafenib may affect Akt signaling due to its effects in downregulating the level of p-Akt. In conclusion, this study shows that sorafenib is potentially cytotoxic in HepG2 cells. This may be, at least, partially attributed to the induction of cell cycle arrest, the increase in the level of active caspase-3 as well as the downregulation of the p-Akt level.

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الملخص العربي

السمية الخلوية لعقار "سورافينيب" فى الخلايا السرطانية الكبدية الآدمية

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يعتبر سرطان الكبد النوع الرئيسي من الأمراض الخبيثة التى تصيب الكبد ويتميز بارتفاع معدلات الوفيات في جميع أنحاء العالم. وتعد معظم أورام الكبد مقاومة بطبيعتها للعلاج الكيميائي وعلى الرغم من التقدم الهائل في العلاج الكيميائي للسرطان، يظل علاج هذة الأورام يمثل تحدياً كبيراً.

ومؤخراً، تم اعتماد عقار"سورافينيب"، وهو مثبط لعديد من الكيناز، لعلاج سرطان الكبد المتقدم. وقد هدفت الدراسة الحالية إلى استكشاف المزيد عن أنشطة السمية الخلوية المحتملة لعقار"سورافينيب" فى خلايا "هيب جى-٢" وكذلك استكشاف آلياته الكامنة. وبالتالى، تمت معالجة خلايا "هيب جى-٢" بتركيزات مختلفة من عقار "سورافينيب". ومن منحنيات البقاء الملائمة تم حساب التركيز المانع لنمو ٥٠% من الخلايا. كما تم التحقق من تأثير عقار "سورافينيب" على دورة الخلية وموتها المبرمج وتكاثرها. وقد حفزعقار"سورافينيب" السمية الخلوية فى خلايا "هيب جى-٢". و هذا يمكن أن ينسب جزئياً إلى زيادة الموت المبرمج عن طريق زيادة مستوى نشاط كاسبيز-٣. علاوة على ذلك، فإن عقار "سورافينيب" حفز إيقاف دورة الخلية وكانت له أنشطة مضادة للتكاثر من خلال تقليل مستوى p-Akt.