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Suppression of hepatocellular carcinoma growth in mice by the alkaloid coccidiostat halofuginone

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Abstract

Halofuginone, a widely used alkaloid coccidiostat, is a potent inhibitor of collagen α1 (I) and matrix metalloproteinase 2 gene expression. Halofuginone also suppresses extracellular matrix deposition and fibroblast proliferation. It was recently shown to be effective in suppression of bladder carcinoma and glioma. This study sought to evaluate the effect of treatment with halofuginone on growth of hepatocellular carcinoma (HCC) in mice. Athymic Balb/c mice were injected subcutaneously with 10^7 human hepatoma cells (Hep3B), followed by treatment with halofuginone administered in the diet (750 μg/kg) starting on day 3, before tumour innoculation. The control group was received a normal diet. Mice were followed for survival, tumour volume and serum α-fetoprotein (αFP). The mechanism of the anti-tumour effect of halofuginone was determined in vitro by assessing tumour cell growth, and by measuring the serum concentrations of interferon-γ (IFNγ) and interleukin 2 (IL2). Halofuginone treatment induced almost complete tumour suppression in treated mice. Mortality rates were 10% and 50%, in halofuginone-treated and control mice, respectively ($P < 0.001$). No visible tumour was observed in treated mice, as compared with a 364 mm^3 tumour in control mice. Serum αFP were 0.1 and 212 ng/ml in treated and control mice, respectively ($P < 0.005$). Halofuginone significantly inhibited HCC proliferation in vitro. Maximal inhibition of 64% of tumour cell growth was observed at a concentration of 10^{-8} M. The anti-tumour effect was mediated via a significant increase in IFNγ and IL2 (90 vs. 35, and 210 vs. 34 pg/ml in treated and control groups, respectively, $P < 0.005$). Treatment with halofuginone effectively suppressed the progression of HCC in mice. This effect may be associated with a direct anti-tumour effect, and/or enhancement of a systemic immune response.

1. Introduction

Hepatocellular carcinoma (HCC) is one of a group of neoplasms that are resistant to systemic chemotherapy [1]. The incidence of HCC in the Western world varies between 0.15% and 0.69%, accounting for about 1% of all cancers. The median survival for patients with unresected tumours are reported to be between three and four months. Although palliative treatments such as radioablation, chemoembolization, and intratumour alcohol injection have prolonged survival, the prognosis in general remains poor for the majority of patients [2–4].

Overall the risk of HCC is about 40 times greater in patients with cirrhosis than in patients with a normal liver [2,5]. It was recently demonstrated in an orthotopic mouse model that the characteristic behaviour of HCC in intrahepatic metastasis was more evident in cirrhotic rather than normal liver. Primary liver cancer will eventually develop in 2% of patients with alcoholic cirrhosis compared with 10% of patients with the macronodular form of cirrhosis [2,3].

Halofuginone is a low molecular-weight quinazolinone alkaloid (495 kDa, structure presented in the Merck Index) isolated from the plant *Dichroa febrifuga*. It is widely

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used as a coccidiostat in chickens and turkeys, and is a potent inhibitor of collagen type α1 (I) gene expression and extracellular matrix (ECM) deposition [6]. Specific inhibition of collagen type I synthesis was demonstrated in a broad range of cell types of chicken, mouse, rat and human origin, both in vitro and in experimental animals [7–11]. The effect of halofuginone on thioacetamide (TAA)-induced liver fibrosis in rats has also been evaluated [12]. In the control rats, the hepatic stellate cells (HSC) did not express smooth muscle actin, collagen type I gene, or tissue inhibition of metalloproteinases-2 (TIMP-2), suggesting that they were in their quiescent state. When treated with TAA, the livers displayed large fibrous septa populated by smooth muscle actin-positive cells expressing high levels of the collagen α1 (I) gene and containing high levels of TIMP-2, all of which are characteristic of advanced fibrosis. Halofuginone given orally before fibrosis induction prevented the activation of most of the stellate cells and the remaining cells expressed low levels of collagen α1 gene, resulting in low levels of collagen [12]. The levels of TIMP-2 were almost the same as in the control livers. When given to rats with established fibrosis, halofuginone induced almost complete resolution of the fibrotic condition. Levels of collagen, collagen α1 [1] gene expression, TIMP-2 content, and smooth muscle actin-positive cells were similar in treated and control rats [13].

Halofuginone has also been shown to inhibit tumour progression in two models of mouse bladder carcinoma: (a) tumours formed by subcutaneous transplantation of MBT2-t50 murine bladder carcinoma cells into syngeneic mice; and (b) bladder carcinoma induced by BBN, a bladder-specific carcinogen [14]. A strong anti-cancerous effect of halofuginone was observed in both experimental models, even when the halofuginone treatment was initiated at relatively advanced stages of tumour development. The anti-tumour effect of halofuginone was attributed to its action on several critical steps in primary tumour progression, such as angiogenesis, stromal support and cell proliferation. Moreover, halofuginone also suppressed transcription of the *MMP-2* gene, associated with a marked decrease in ECM invasion in vitro and lung colonization by bladder carcinoma cells [15,16].

The aim of the present study was to determine the anti-tumour effect of halofuginone on human HCC in vitro and in vivo, and to examine the influence of interferon-γ (IFNγ) and interleukin 2 (IL2) secretion on this process.

2. Materials and methods

2.1. Animals

Male athymic Balb/c mice, eight weeks old, were purchased from Jackson Laboratories USA. All animals were kept under laminar-flow hoods in sterilized cages, and were given irradiated food and sterile acidified water. All experiments were carried out according to the guidelines of the Hebrew University – Hadassah institutional committee for care and use of laboratory animals, and with the committee's approval.

2.2. Cell cultures

The human hepatoma cell line Hep-3B was grown in culture as monolayers in medium supplemented with non-essential amino acids and 10% heat-inactivated fetal bovine serum, as described earlier [17,18].

2.3. Tumour transplantation in athymic mice

Mice were conditioned with sublethal radiation (600 cGy). Twenty-four hours after irradiation, animals were injected subcutaneously into the area overlying the abdomen with 10^7 human hepatoma Hep-3B cells, as described in detail elsewhere [18].

2.4. Experimental groups

Two groups of mice (eight animals each) were studied. Halofuginone was provided by Roussel-Uclaf (Paris, France). The experimental group was treated orally with halofuginone at a concentration of 3 parts/10^6 starting at day 3 before tumour innoculation. The control group received a regular diet.

2.5. Follow up of tumour growth

Recipient mice were followed for survival and tumour growth was monitored by tumour volume (V), using calipers to measuring the length (L) and the width (W) of the tumour, and the equation $V = LW^2/2$. Measurements of serum α-fetoprotein (AFP) were also taken. Blood samples were obtained on days 0, 7, 17, and 41. Serum was separated and frozen at −20 °C, then assayed by a commercial solid-phase radioimmunoassay (Bridge Serono, Italy) and expressed in ng/ml.

2.6. Tumour cell proliferation

Cells were seeded in Dulbecco modified Eagle medium containing 10% fetal calf serum at a density of 3×10^3 cells/16-mm well of a 24-well plate, in quadruplicate. The medium was replaced 24 h after seeding, and the cells were cultured for seven days in the absence or presence of increasing concentrations of halofuginone, ranging from 10^{-12} to 10^{-8} M. On each day after seeding, cells of four wells were dissociated with trypsin/EDTA and counted with a Coulter counter (Coulter Electronics Ltd.)

2.7. Cytokine production

In an attempt to evaluate the effect of halofuginone on IFNγ and IL2 production, serum concentrations were assessed by enzyme-linked immunoassay according to manufacturer's instructions (R&D Systems, USA). All mice in experimental and control groups were bled on days 0, 7, and 17 for IL2, and on days 0, 17, and 41 for serum IFNγ.

3. Results

3.1. Halofuginone suppressed in vitro HCC growth

The anti-tumour effect of halofuginone may be attributed to a direct inhibition of tumour cell proliferation. As shown in Fig. 1, culturing the Hep-3B HCC cell line in the presence of 10^{-12}–10^{-8} M halofuginone induced a $33 \pm 4\%$–$64 \pm 6\%$ inhibition in cell growth. The maximal effect was observed at 10^{-8} M (64% inhibition, $P < 0.005$; Fig. 1). Halofuginone did not exert a direct toxic effect on the cells as cell viability was not affected by the treatment (cell viability ranged from $77 \pm 8\%$ to $98 \pm 7\%$).

3.2. Halofuginone treatment suppressed in vivo tumour growth and serum AFP in tumour-bearing mice

Halofuginone treatment induced significant tumour suppression. No visible tumour was observed in treated mice. Tumour volume was 364 ± 15 mm^3 by week 3 of the experiment as compared with 0 in the control and halofuginone-treated groups, respectively ($P < 0.005$; Fig. 2).

Serum AFP concentrations did not rise in halofuginone-treated mice (Fig. 4). They decreased from 380 ± 29 to 3.8 ± 0.7 ng/ml, from 212 ± 31 to 0.1 ± 0.05 ng/ml, and from 346 ± 45 to 10.3 ± 1.5 ng/ml, in control and halofuginone-treated mice, on days 7, 17, and 41, respectively ($P < 0.001$; Fig. 3).

3.3. Halofuginone treatment increased survival of tumour-bearing mice

Halofuginone treatment significantly improved the survival of tumour-bearing mice. A 90% survival rate was observed in treated mice compared with 50% mortality in the controls ($P < 0.001$; Fig. 4).

3.4. Halofuginone increased serum IFNγ and serum IL2

As IFNγ and IL2 are two of the important mediators in an anti-tumour effect, their concentrations were assessed in halofuginone-treated and control mice. No significant differences were observed on day 0 between the two groups. Treatment with halofuginone significantly increased the serum IFNγ and IL2. IFNγ to 90 ± 8.4 pg/ml in treated mice as compared with 35 ± 4.3 pg/ml in the control group, on day 17 (Fig. 5; $P < 0.005$). Similarly, serum IL2 increased significantly to 210 ± 19 pg/ml as compared with 34 ± 3.8 pg/ml in treated and control mice, respectively, on day 17 (Fig. 6; $P < 0.005$).

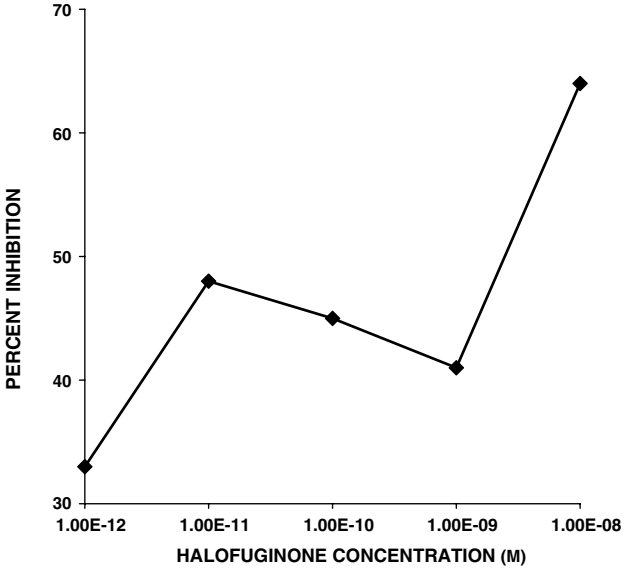

Fig. 1. Halofuginone suppressed in vitro hepatocellular carcinoma growth: culturing of Hep-3B HCC cell line in the presence of 10^{-8}–10^{-12} M halofuginone induced a decrease in growth. The maximal effect was observed at 10^{-8} M.

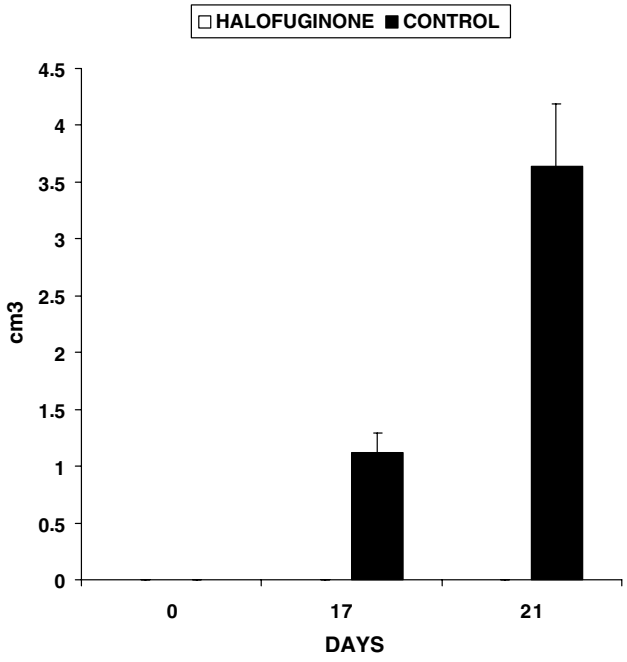

Fig. 2. Effect of treatment with halofuginone on tumor growth in tumor bearing mice: a significant decrease in tumor volume was observed in halofuginone treated vs. non-treated controls.

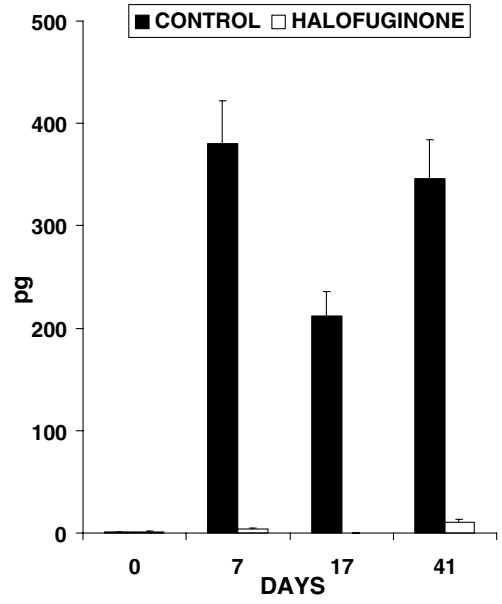

Fig. 3. Effect of treatment with halofuginone on serum alpha feto protein levels (AFP) levels in tumor bearing mice: a significant decrease in AFP levels was observed in halofuginone treated vs. non-treated controls.

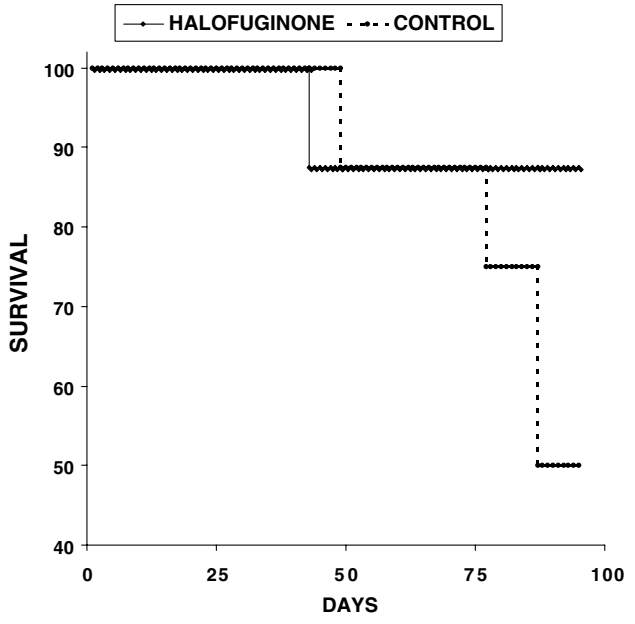

Fig. 4. Halofuginone treatment significantly improved survival in tumor bearing mice. A 90% survival was observed in treated mice compared with 50% mortality in controls.

4. Discussion

The results of the present study show that treatment with halofuginone significantly suppressed HCC growth in vitro and in vivo, and increased the survival of HCC-bearing treated mice. The anti-tumour effect may be attributed to direct and systemic anti-tumour activities.

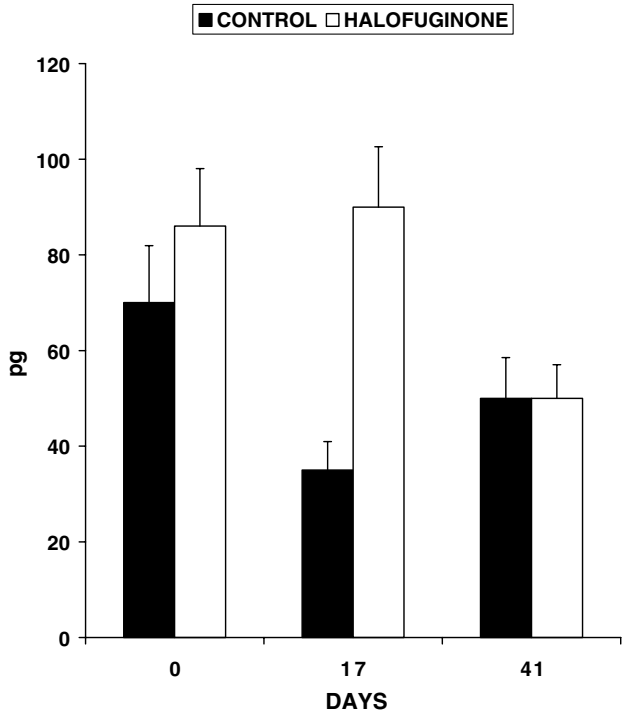

Fig. 5. Effect of treatment with halofuginone on IFNγ serum levels in tumor bearing mice: a significant increase in IFNγ serum levels was observed on day 17 in halofuginone treated vs. non-treated controls.

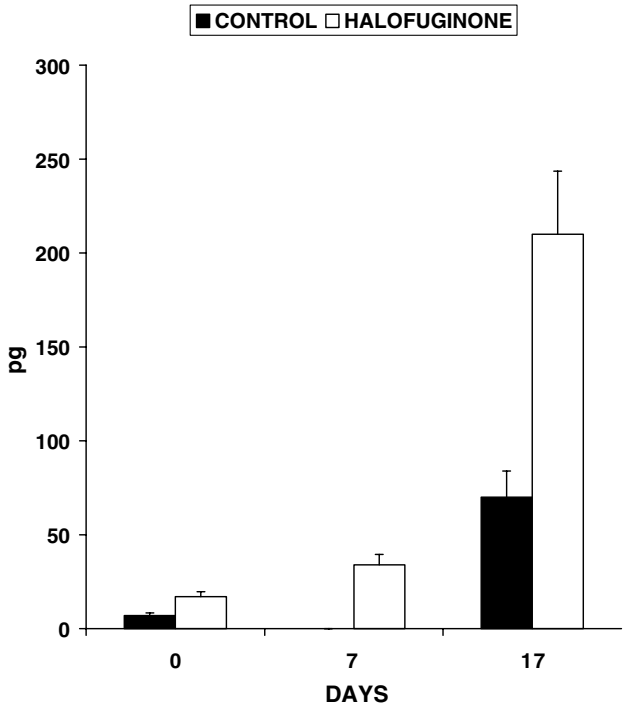

Fig. 6. Effect of treatment with halofuginone on IL2 serum levels in tumor bearing mice: a significant increase in IL2 serum levels was observed on day 17 in halofuginone treated vs. non-treated controls.

In previous studies, we have demonstrated that halofuginone, a low molecular-weight quinazolinone-derived alkaloid isolated from the plant *D. febrifuga* and

widely used for over 20 years as a coccidiostat in chickens and turkeys, suppresses collagen type α1 (I) gene expression and ECM deposition [6–11]. Specific inhibition of collagen type I synthesis was demonstrated in a broad range of cell types of chicken, mouse, rat and human origin, both in vitro and in experimental animals. Halofuginone was also shown to inhibit transforming growth factor (TGF)-β-stimulated collagen α1 (I) synthesis by human skin fibroblasts [8–15]. Moreover, exposure to halofuginone was found to inhibit the deposition of ECM by vascular smooth muscle and kidney mesangial cells [9,10,19].

The anti-HCC effect of halofuginone reported in the present study is consistent with several recent publications describing the anti-tumour capacity of this compound against several malignancies including bladder carcinoma, C6 glioma and prostate cancer [13–15,20]. This anti-tumour effect against primary tumours was previously attributed to the inhibition of cell proliferation, extracellular matrix and collagen deposition, and to anti-angiogenetic properties. Several possible mechanisms for tumour growth suppression by halofuginone should be considered. In previous studies, we reported that halofuginone exerts an anti-proliferative effect on various tumour types [13–15,20]. Nevertheless, the observed therapeutic effect of halofuginone cannot be attributed solely to its direct anti-proliferative activity. Previous data indicate that the role of halofuginone in tumour suppression is more complex than that of merely anti-proliferative cytostatic agents. Rather, an effect on tumour angiogenesis and stromal support seems to play a decisive role.

Furthermore, halofuginone was previously demonstrated to strongly inhibit the activity of matrix metalloproteinase (MMP)-2 [13]. The invasive behaviour of neoplastic cells and their ability to metastasis to distant sites are multistep processes that include the detachment of the cells from the original tumour mass, their attachment to ECM binding sites, the degradation of ECMs, and their migration into surrounding tissues [21–24]. One of the rate-limiting steps in the metastatic cascade is the activity of MMPs degrading a variety of ECM proteins [22,23]. A central role in this process is played by MMP-2, which cleaves primarily type IV collagen in the basement membrane [25,26]. We have previously demonstrated that halofuginone inhibits MMP-2, leading to a marked decrease in both ECM invasion and lung metastasis. This inhibition was consistent with the suggestion of a possible common transcriptional regulation of ECM components and ECM-degrading enzymes [13–15].

In addition, common regulatory pathways for the production, deposition and degradation of ECM components have been described, mainly through TGF-β1-mediated transcriptional effects [27]. TGF-β1 is a pluripotent regulator of a variety of cellular activities including cell growth, differentiation, and synthesis of ECM constituents [28–30]. In fact, TGF-β_1 promotes the expression of genes encoding several types of collagen (i.e. I, III, and V) and collagen-degrading enzymes such as MMP-2 [31–33], which suggests a possible common regulatory element in the transcriptional control of ECM proteins (i.e. collagen type I) and MMP-2. It was recently demonstrated that the inhibition of TGF-β_1 by halofuginone is via blockage of the TGF-β_1 signalling pathways, mediated by inhibition of the phosphorylation and subsequent activation of Smad3 [34].

In the present study, we have shown an additional possible mechanism for the anti-tumour effect of halofuginone. Halofuginone treament significantly increased IFNγ and IL2 concentrations. The effect of halofuginone on the cytokine cascade has never, to the best of our knowledge, been studied before. Hepatic fibrosis is currently perceived as a programmed response to injury [35,36]. Stellate cell activation is a central feature of all forms of liver injury. It is therefore conceivable that part of the anti-fibrotic effect of halofuginone in an animal model of liver fibrosis is mediated via a systemic effect on cytokine production. Several studies support the role of immune modulation in hepatic fibrosis and suggest that Th1/Th2 cytokine subsets can modulate the fibrotic response to injury ([36,37]. Liver fibrosis induced with carbon tetrachloride was shown to be associated with alterations in Th1/Th2 cytokine mRNA. Fibrogenesis was significantly modified in T- and B-cell-deficient BALB/c and C57BL/6 SCID mice compared with their wild-type counterparts. Fibrogenic BALB/c mice exhibited a Th2 response during the wounding reaction, whereas C57BL/6 mice displayed a Th1 response, suggesting that hepatic fibrosis is influenced by different T-helper subsets [38–40]. Moreover, mice lacking IFNγ which default to the Th2 cytokine pathway exhibited more pronounced fibrotic lesions than did wild-type animals [36]. Finally, a shifting of the Th2 response towards a Th1 response by treatment with IFNγ ameliorated fibrosis in BALB/c mice [39–43]. Stellate cell activation has been observed in primary and metastatic tumours. In a rodent model, melanoma cells activate stellate cells in vivo and in culture, stimulating proliferation, matrix production and matrix degradation through increased MMP2 [44].

Increased production and/or activity of cytokines are critical for both the autocrine and paracrine perpetuation of stellate cell activation. Cytokines play a major part in the development of hepatic fibrosis [36–38]. Cytokines may be pro- or anti-fibrogenic, autocrine or paracrine [36,37]. Virtually all features of stellate cell activation can be attributed in part to autocrine cytokines. These include TGFβ_1, platelet-derived growth factor, fibroblast growth factor, hepatocyte growth factor, platelet-activating factor and endothelin 1. Anti-inflammatory cytokines produced by stellate cells have

also been identified. Stellate cells express IL10 upon activation, and the autocrine effects of this cytokine include inhibition of collagen production [37]. Activation of stellate cells causes enhanced autocrine expression of IL10, which has a negative autoregulatory effect on collagen production by stellate cells [37]. This is mediated in part by the transcriptional inhibition of α1 (I) collagen and the stimulation of collagenase expression. Therefore, it is conceivable that halofuginone, which is a very potent inhibitor of collagen type I synthesis and gene expression as well as TGFβ and Smad 3, exerts its effect via the suppression of cytokine production by stellate cells. Indeed, we were able to demonstrate that halofuginone inhibited collagen production and collagen α1 (I) gene expression in immortalized rat stellate cells in vitro [12]. Liver cancer is more common in patients with fibrosis, which is regarded as a first step towards carcinogenesis. Cytokines were shown to play a part in both fibrosis and liver carcinogenesis, and therefore may serve as the common mediator for both [42]. The anti-tumour effect of halofuginone may be mediated via modulation of both cytokine-mediated fibrosis and tumorigenesis.

Tumours can escape from immunosurveillance by releasing immunosuppressive factors, leading to dysfunction of T-cells, the induction of T-cell apoptosis, and the secretion of cytokines such as IL10 [45–47]. In the present study, we were able to demonstrate an increase in anti-tumour cytokines, IL2 and IFNγ. These studies suggest that halofuginone may direct the immune response towards a Th1 pattern and/or mediate the activation of anti-tumour natural killer (NK) cells or NKT cells. It may therefore be of interest to assess the effect of halofuginone on the cytotoxic and proliferative capabilities of NK and cytotoxic T-lymphocytes.

These features make halofuginone a likely candidate for additional studies in animal models of hepatocellular tumour progression, toward clinical trials in man. Altogether, our results suggest that halofuginone may exert a profound anti-HCC effect through a combined action on several critical stages in tumour progression.

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