original research report

Arsenic trioxide plus cisplatin/interferon α-2b/doxorubicin/capecitabine combination chemotherapy for unresectable hepatocellular carcinoma

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BACKGROUND AND OBJECTIVES: The failure of existing treatments for liver cancer has recently been attributed to the existence of cancer stem cells, which are difficult to kill using current drugs due to their chemoresistant properties as well as their ability to stimulate neoangiogenesis. The aim of the current study was to evaluate in vitro the antitumor efficacy of arsenic trioxide in combination with conventional chemotherapy, as proposed by the concept of "differentiation therapy" in anticancer research.

MATERIALS AND METHODS: Cancer stem cells showed enhanced chemoresistance to cancer drugs (carboplatin and doxorubicin) and had the ability to exclude rhodamine 123 dye, proving the existence of the multidrug resistance efflux pump. Arsenic trioxide was added prior to a tyrosine kinase inhibitor or to a slightly modified Piaf regimen with capecitabine replacing 5-fluorouracil. We also compared both cancer and normal stem cell lines with the hepG2 non-stem liver cancer cell line to investigate the differences between differentiated and more anaplastic cells. Molecular characterization (immunocytochemistry and rt-PCr analysis) of all the cell lines was carried out.

RESULTS: Initially, the cells had a high proliferative potential, even when cultured in a medium supplemented with cytostatics, eliminated rhodamine 123 immediately in culture and also formed spheroids in suspension. The molecular characterization showed the expression of albumin, α1-antitrypsin, α-fetoprotein, citokeratin-18, telomerase, CD90 and CD133. Low concentrations of arsenic trioxide lead to morphologic differentiation and differentiation-associated cytochemical features, like increased sensitivity to cytostatic drugs.

CONCLUSION: Our study suggests that arsenic trioxide sensitizes liver stem-like cancer cells to conventional chemotherapy. Still, further studies on animal models will be needed before we implement this idea in human clinical trials.

Hepatocellular carcinoma (HCC) is a very common neoplasia and there has been little improvement in 5-year survival in the last two decades (with a median survival of less than 1 year in the United States or Europe) despite the development of new diagnostic techniques such as ultrasonography, CT, MRI and angiography, and advancements in therapeutic modalities, such as surgical resection, radiofrequency ablation, percutaneous ethanol injection, transcatheter arterial chemoembolization, radiotherapy and intra-arterial infusion via implantable drug delivery systems. HCC chemotherapy includes patients with advanced-stage tumors signified by portal vein invasion, metastasis or failure of other treatment options and is usually limited to persons with Eastern Cooperative Oncology Groups performance status of 0 to 2 and intact hepatic function (Child-Pugh A or early B). Even if the results are mainly palliative, combination therapy

may improve the response rate, and the main trends include PIAF (cisplatin, doxorubicin, 5-fluorouracil and Interferon α-2b) and tyrosine kinase inhibitors.[1,2]

The failure of existing treatments for cancer has recently been attributed to the existence of cancer stem cells (CSCs), which are difficult to kill using current drugs due to their chemoresistant properties as well as their ability to stimulate neoangiogenesis. These properties contribute to tumor recurrence and treatment resistance in advanced cases via activation of ABC transporters, as well as Notch and Hedgehog-Gli1 signaling pathways involved in self-renewal and tumorigenicity.[3] The American Association for Cancer Research sugests that successful therapies should target CSCs by completely eliminating them via inhibition of the self-renewing stem cell state or by inducing differentiation of CSCs into non-tumorigenic cells, more sensitive to conventional therapy.[4] The differentiation therapy hypothesis has recently been investigated by Ito et al, by using the inorganic arsenite, arsenic trioxide As2O3 (ATO), in the treatment of leukemia.[5] ATO treatment significantly decreased the number of quiescent leukemia stem cells without inducing apoptosis, this result being confirmed by Kobayashi et al, who treated a patient with acute promyelocytic leukemia with arsenic plus conventional chemotherapy.[6] If acute promyelocytic leukemia can be considered a model disease for anticancer research,[7] we investigated the in vitro effects of low concentrations of ATO on CSCs isolated from solid tumors.

MATERIALS AND METHODS

Cell culture protocols

Cancer stem cells were isolated from a hepatocellular carcinoma biopsy, as previously described.[3] CSCs, along with normal liver stem cells and the non-stem tumor cell line HepG2 (both kindly provided by the Professor Carmen Mihu, MD, PhD, Department of Histology, Iuliu Hatieganu University of Medicine and Pharmacy from Cluj Napoca, Romania) were maintained in Ham's F-12 and Dulbecco's Modified Essential Medium at 1:1 ratio, supplemented with 10% fetal calf serum (FCS), 100 U/mL penicillin and 100 μg/mL streptomycin (all from Sigma Aldrich, St Louis, MO, USA) in a 37 °C humidified incubator with a mixture of 95% air and 5% carbon dioxide. All experiments were performed on exponentially growing cells, with a doubling time of approximately 24 to 36 hours.

Multidrug resistance assay

To assess the resistance of cells isolated from hepatocellular carcinoma to chemotherapy, cells were seeded at 3000 cells/well in 96-well plates. The culture media was DMEM/F12 supplemented with growth factors. Cancer stem cells were compared with HFL human lung fibroblasts (European Collection of Cell Cultures, Budapest, Hungary) and with MLS human ovarian tumor cells (courtesy of Dr Yael Schiffenbauer, Weizmann Institute of Science, Rehovot, Israel). Six hours later, all cells were treated with 5 μg/mL carboplatin and 5 μg/mL doxorubicin, concentrations higher than normally used in the clinic. After 24 and 48 hours, the relative cell number was determined by standard MTT assay. The MLS tumor ovarian cell line was used as another control of more differentiated tumor cells, apart from the HepG2 cell line.

Rhodamine 123 efflux assay

Hepatic cancer stem cells, MLS ovarian tumor cells and HFL human fibroblasts were seeded in specific culture media supplemented with FCS, non-essential amino acids, L-glutamine and antibiotics, at 20×10^4 cells/mL. Cells were stained with 10 μM rhodamine 123 and then incubated for three hours at 37°C and 7% CO_2. After culture, all cell types were washed three times with PBS before intracellular fluorescence was measured using a BioTek Synergy 2 fluorescence microplate reader (Winooski, VT, USA).

Reactives and cytostatics

Arsenic trioxide (kindly provided by Professor Luminița Silaghi-Dumitrescu, Department of Inorganic Chemistry, Babeș-Bolyai University from Cluj Napoca, Romania) was added at a concentration of 0.5 μM prior to a tyrosine kinase inhibitor or to a slightly modiffied PIAF regimen with capecitabine replacing 5-fluorouracil, as according to the results of von Delius et al.[8] Sorafenib (a tyrosine kinase inhibitor) was used at a concentration of 4.6 μg/mL, doxorubicin at 0.5 μg/mL, cisplatin at 0.25 μg/mL, capecitabine at 30 μg/mL and interferon α-2b at 2 μg/mL, as used in the clinic.

Proliferation assay

Cell survival was assessed using the MTT assay, as previously described.[9] For 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays, cells in monolayer culture were irradiated, incubated in DMEM media supplemented with 10% FCS, 100 U/mL penicillin and 100μg/mL streptomycin before being washed twice with phosphate buffer solution (PBS). Cells were then incubated with trypsin-EDTA, resuspended in culture medium with FCS, counted and plated in 100 μL media at 15×10^3 cells/well in 96-well microtiter plates. After 24 hours, cells were washed and treated with a concentration of 0.5 μM ATO before adding cytostatic

Table 1. Primers used for the RT-PCR characterisation of the cancers stem-like cells, normal hepatic stem cells and HepG2 hepatic tumor cells.

Primers	Sequence (5'→3')	Size (bp)	Melting temperature (°C)
Oct-4	aggagtcccaggacatcaaag tcgtttggctgaataccttc	146	56
Rex-1	atggctatgtgtgctatgagc ctcaacttcctagtgcatcc	447	50
TERT	catcatcaaaccccagaacac caaacagcttgttctccatgt	371	55
Nanog	attataaatctagagactccag tcctgaataagcagatccatg	407	52
Sox-2	aagcgctttttttgatcctgattc accacaccatgaaggcattcatg	363	57
β2-microglobulin	actccaaagattcaggtttactc catgatgctgcttacatgtc	302	52
HLA-DRα	ttgaagaatttggacgatttg aaactcccagtgcttgagaagag	407	54
Vimentina	ttcagagagaggaagccgaaaac tttaagggcatccacttcacag	422	56
c-kit (CD117)	ttcagcgagagttaatgattctg tgtattcacataaacattaaatg	400	50
Thy-1 (CD90)	aaagaagcacgtgctctttggc actcagagaagtaggatctctg	379	56
GATA-4	cacaagatgaacggcatcaac cagacatcgcactgactgagaa	439	56
GAPDH	acaactttggtatcgtggaa aaattcgttgtcataccagg	458	50

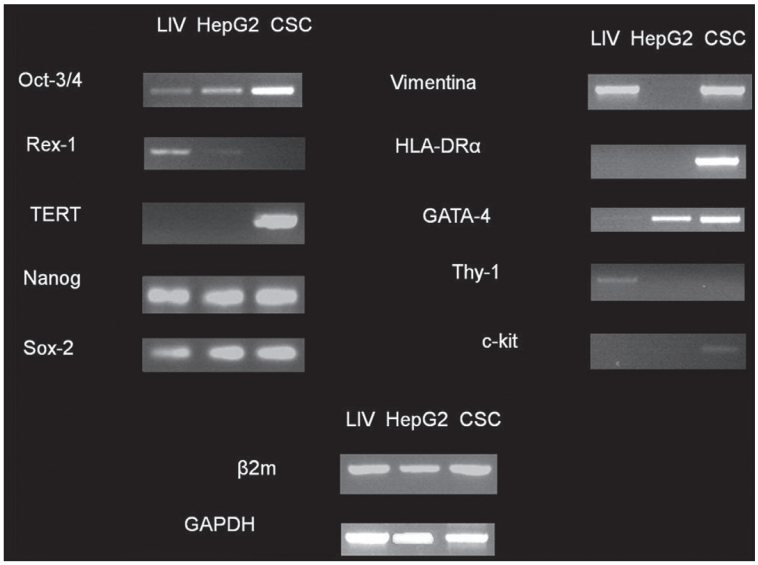

Figure 1a. Expression levels of certain liver specific genes and the catalytic subunit of the telomerase enzyme as determined by RT-PCR.

drugs. Absorbance of the MTT was measured at 492 nm using the fluorescence microplate reader.

Immunocytochemical staining

Hepatocellular carcinoma derived adherent cells were labeled with anti-human antibodies and fixed with 4% paraformaldehyde for 20 minutes. After blocking with Bovine Serum Albumine 10% (Sigma Aldrich), cells were incubated overnight with the following primary antibodies: CD90 and CD133, diluted 1:50. For cell staining with secondary antibodies, the protocols used fluorescein isothiocyanate (FITC) Goat anti-mouse IgG and IgM phycoerythrin (PE) goat anti-mouse IgG or Texas red goat anti-mouse IgG. Cells nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI). The fluorescent cells were visualized with an inverted phase Zeiss Axiovert microscope using filtres of 488 546 and 340/360 nm.

RNA extraction and RT-PCR analysis

Total RNA was isolated from hepatic cancer stem-like cells, from liver mesenchymal stem cells and from the HepG2 cell line. RNA isolation was performed from subconfluent monolayers of adherent cells plated on 6-well dishes, using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. 1 μg of total RNA was used for reverse transcription with the ImProm Reverse Transcription System (Promega, Madison, WI, USA). Only mRNA was transformed into cDNA by using oligo-dT primers in the reaction mixture, together with: AMV reverse-transcriptase 15 u/μg; buffer solution (10 mM Tris-HCl, pH=9.0; 50 mM KCl; 0,1% Triton X-100); dNTP solution, 1 mM each; MgCl2 5mM; recombinant ribonuclease inhibitor 1 u/μL; ultrapure nuclease-free water. The cDNA was amplifi ed using GoTaq PCR Core System II (Promega). The primers used were designed according to the corresponding human genes, as shown in **Table 1**. Amplification reactions were performed on a Techne TC3000 thermal cycler (Bibby Scientific Ltd, Staffordshire, United Kingdom) at 95°C for 30 seconds, 56°C for 1 minute, and 72°C for 2 minutes for 40 cycles. The PCR products were then separated by electrophoresis on 2% agarose gell and photographed with a UV transilluminator.

Statistical significance was evaluated using one-way analysis of variance (ANOVA), with a 95% confidence level using GraphPad Prism 5 statistics program (GraphPad, San Diego, CA, USA). All experiments were performed in triplicate.

RESULTS

Molecular characterisation

The RT-PCR analysis shows that the stem-like cells isolated from the hepatocellular carcinoma biopsy express markers proven to be specific to early hepatic progenitors (**Figure 1a**). Beta-2 microglobulin and GAPDH were used as controls. Darwinian selection of the cancer stem-like cell subpopulation was later on seen in culture, confirming the results found in the clinic after the initial tumoral relapse, when the disease continued to progress despite multimodal aggressive oncology treatments. The expression of stem cell-specific markers was also confirmed by immunocytochemistry staining, which proved the presence of the stem cell-characteristic markers CD 90 and CD133 (**Figures 1b, c**).

Stem-like tumor cells are resistant to conventional chemotherapy

Because rhodamine 123 uses the same pathways to pass through the membrane as conventional drugs used in oncologic treatments, by measuring the optical density (OD) of this fluorescent substance, we were able to determine indirectly whether the cell population isolated from HCC expresses the proteins responsable for multidrug resistance. Hepatic cancer stem cells were compared with both the HFL human fibroblast cell line and to MLS ovarian tumor cell line (**Figure 2**).

To examine whether hepatic tumor cells possessed a hypothesized cancer stem cell chemoresistant phenotype, we also assessed the sensitivity of the cells to carboplatin and doxorubicin under stem cell conditions. Compared with HFL fibroblasts and MLS tumor ovarian cells, both carboplatin and doxorubicin optical density IC_{50} values were greater ($P<.05$) (**Figure 3**). These results support a role for these stem-like cells in hepatic cancer chemoresistance (i.e. failure to eradicate progenitors resulting in tumor regrowth).

Clonogenic survival after treatment

The concentration used was 0.5 µM, readily and safely acheivable in patients. Apart from the previously proven resistance to very high concentrations of carboplatin and doxorubicin, cancer stem cells are also resistant to both therapeutic treatments with sorafenib and the modified PIAF regimen, with capecitabine replacing 5-fluorouracil. Normal stem cells are also resistant, in comparison with the differentiated tumor cell line HepG2. When adding ATO prior to treatment, the results show a reduced survival of tumor cells in a dose-dependent manner, at 24, 48 hours and 72 hours (**Figures 4a, b, c**). Our

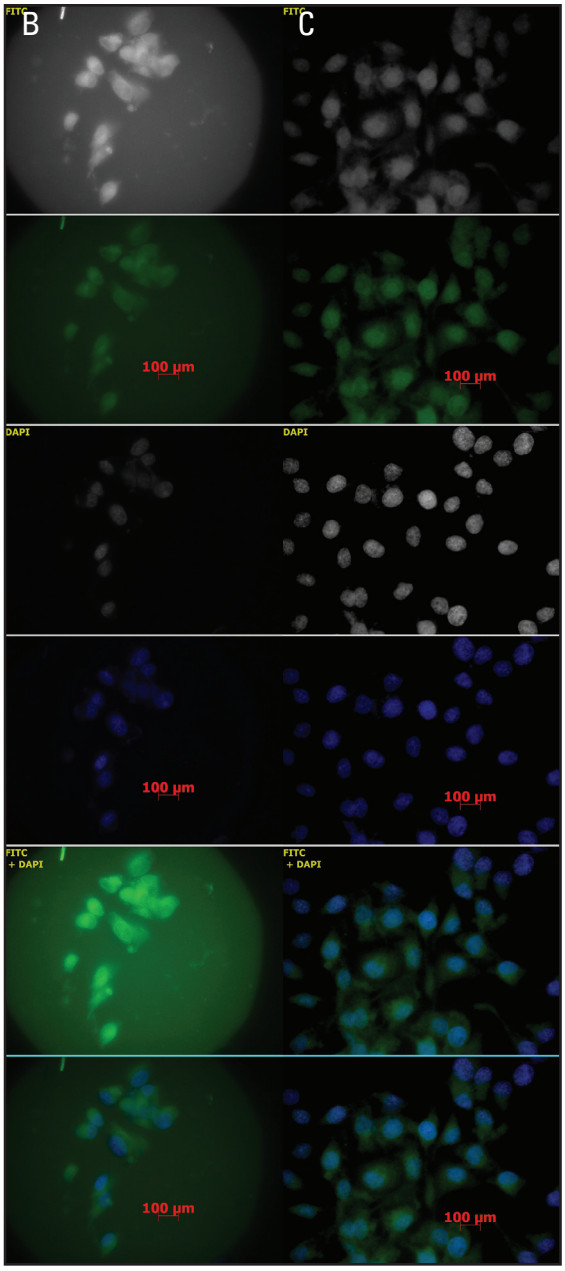

Figure 1b,c. Immunocytochemistry stainings demonstrating that the cells are positive for both CD133 (×100) (B) and for CD 90 (×100) (C).

results demonstrate that the ATO + PIAF regimen is the best combination chemotherapy for hepatocellular carcinoma, superior to both tyrosine kinase inhibitor and only PIAF regimen, in all theree cell lines ($P<.05$).

DISCUSSION

HCC has been a very common disease in Far East Asia, South Africa and Sub-Saharan Africa for several de-

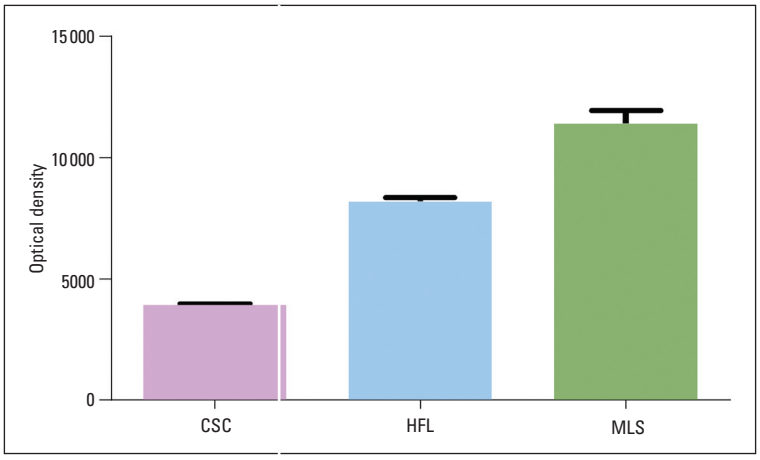

Figure 2. After culture for three hours in a humidified atmosphere at 37°C and 5% carbon dioxide, hepatic cancer cells had the capability for accelerated efflux from the cytoplasma of fluorescent rhodamine 123 using the ABC family proteins. The fibroblasts also expressed, to some extent, this characteristic but the ovarian tumor cells were not resistant, failing to eliminate most of the dye. CSC: cancer stem cells, HFL: human fetal lung fibroblasts, MLS: human ovarian tumor cell line

cades because of the high prevalence of hepatitis B viral (HBV) infection and aflatoxin exposure. In the last few years, a sharp increase of HCC incidence in North America, European Union and Japan has been reported due to hepatitis C viral (HCV) infection, alcohol abuse and non-alcoholic fatty liver disease. Liver cancer is the forth most common neoplasia and the third most common cause of cancer-related death in the world, with incidence rates varying from 2 per 100 000 population in the US to 80 per 100 000 population in China, with an overall 5-year survival rate lower than 5%.[10-11]

The best long-term treatment for patients with HCC is orthotopic liver transplantation, in the case of which eligibility is limited to those meeting the Milan criteria (one nodule <5 cm or maximum three nodules <3 cm each) with a 5-year survival rate after trasnplantation at about 70%, but tumor progression while waiting for a matched donor is the most common cause of drop-out from the transplant list.[12] Thus, the most common treatment, including neoadjuvant treatment for those on the transplant list, is intended to control the progression by methods that include transarterial chemoembolization, selective internal radiotherapy, radiofrequency ablation and percutaneous injection with either ethanol or acetic acid. This treatment is not curative, but reduces the viable tumor volume and slows cancer progression for a while, until resistance appears and treatment becomes useless. The mechanism of HCC resistance is complex, depending on the plasticity of the cell of origin—often a dysfunctional progenitor or cancer stem cell.[13] As most HCC are clonal and thus considered to originate from one progenitor cancer stem cell, modern oncology should focus on targeting this biological entity in order to aim for a cure in hepatic cancer.

As we previously mentioned, the failure of existing treatments for cancer has been recently been attributed to the existence of CSCs, which are difficult to kill using current drugs because of their chemoresistant properties as well as their ability to stimulate neoangiogenesis. Differentiation therapy is a state-of-the-art concept in oncology, only recently reported for leukemia and practically inexistent for solid tumors. Our study is the first in the country and one of the first in international internal medicine reporting the chemosensitization capacity of arsenic trioxide for hepatocellular carcinoma, supporting the pro-differentiation hypothesis in anticancer research.

Arsenium, used in medicine for more than 2400 years,[14,15] was approved by the United States Food and Drug Administration for use in the treatment of relapsed/refractory acute promyeolocytic leukemia because it downregulates Bcl-2 levels, inteferes with the Notch signaling pathway and promotes differentiation followed by apoptosis, arresting cells either in G2M or both G1 and G2M.[16] We also show that low concentrations of ATO lead to morphologic differentiation and differentiation-associated cytochemical features, like increased sensitivity to cytostatic drugs. ATO may have stimulated stem cell differentiation (both normal hepatic stem cells and CSC), thus sensitizing them to chemotherapy. The two stem cell lines are compared with the HepG2 non-stem liver cancer cell line to investigate the differences between differentiated and more anaplastic cells, confirming that an efficient treatment for liver cancer should target the stem cell pool, as the other cancerous cells will eventually undergo apoptosis. Because there are fewer CSC in the G0 state, conventional chemotherapy will be more efficient in the treatment of non-surgical HCC, but further in vivo experiments on laboratory animals and an analysis of absorbtion rate and side effects are required before we can administer arsenic trioxide to patients diagnosed with liver cancer.

In conclusion, human HCC is composed of a hierarchy of mixed tumor cells with different biological properties, including tumor-initiating cells or cancer stem cells. CSCs, with self-renewal and differentiation capability, constitutes a small population of this hierarchy and are thought to give rise to tumor heterogenity and chemoresistance. Thus, CSC eradication is critical to achieve stable remission or even a cure for aggresive malignancies such as liver cancers. One way to eradicate CSCs being obtained through stem cell differentiation

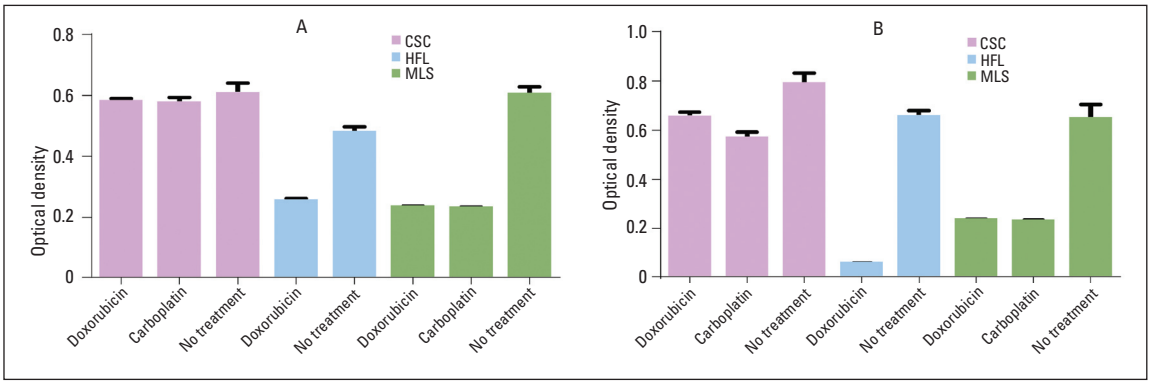

Figure 3. Liver CSC, under stem cell conditions, were highly resistant to conventional chemotherapies. Cells were treated with 5 µg/mL carboplatin and 5 µg/mL doxorubicin for 24 hours (a) and for 48 hours (b). Hepatic tumor cells (both treated and the control group) were compared with fibroblasts and ovarian tumor cells and cell survival was determined by MTT assay.

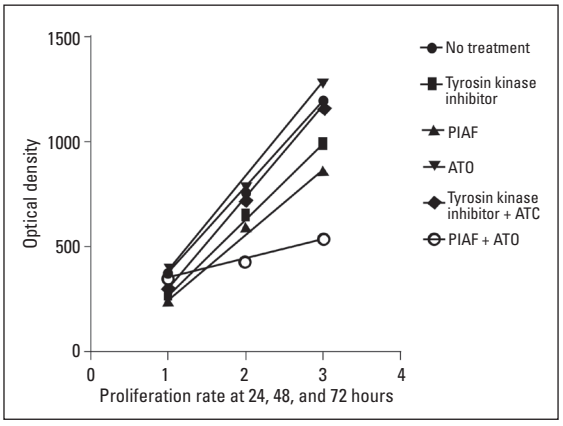

Figure 4a. Survival curves for cancer stem cells and PIAF combination chemotherapy regimen.

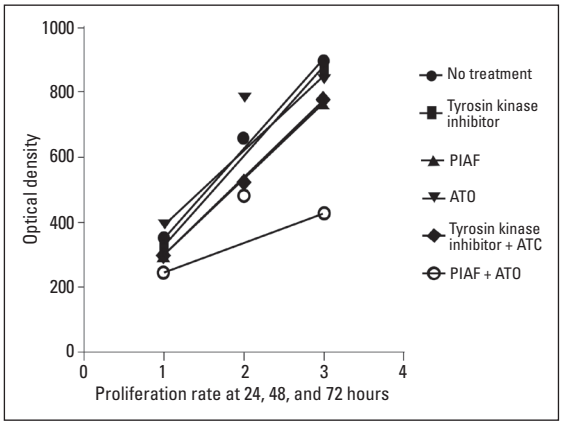

Figure 4b. Survival curves for normal stem cells and PIAF combination chemotherapy regimen.

using pro-differentiation agents such as arsenic trioxide, already used in the clinic for hematologic malignancies. Our results demonstrate for the first time this hypothesis in the potential treatment of hepatocellular carcinoma. This data is critical to realizing the potential for synergy between arsenic trioxide agent chemotherapeutic protocols, such as PIAF, in hope to provide in the near future an enhaced benefit for liver cancer treatment.

Acknowledgments

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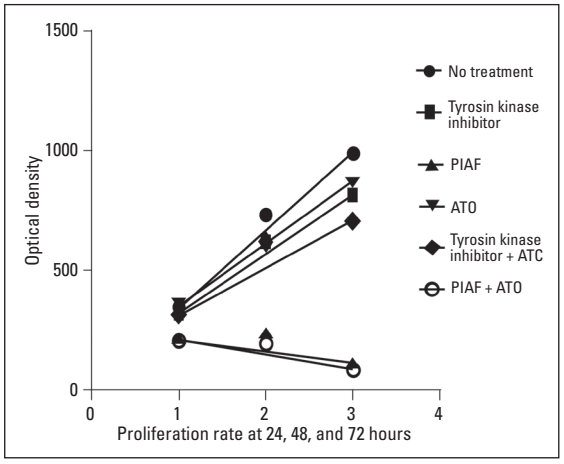

Figure 4c. Survival curves for differentiated hepatic tumor cell line HepG2 and PIAF combination chemotherapy regimen.

Author contributions

Tomuleasa C: designed study, performed experiments and wrote manuscript, Soritau O: performed experiments, Fischer-Fodor E: performed experiments, Susman S: performed experiments, Pop T: performed experiments, Mosteanu O: performed experiments, Acalovschi M: performed experiments, Irimie A: reviewed manuscript, Kacso G: designed study and reviewed manuscript.

Authors declare no conflict of interest.

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