Available online at www.sciencedirect.com
SCIENCE DIRECT
European Journal of Pharmacology 541 (2006) 129–137
www.elsevier.com/locate/ejphar

The new orally active iron chelator ICL670A exhibits a higher antiproliferative effect in human hepatocyte cultures than O-trensox

Karine Chantrel-Groussard [a], François Gaboriau [a], Nicole Pasdeloup [a], René Havouis [b], Hanspeter Nick [c], Jean-Louis Pierre [d], Pierre Brissot [a], Gérard Lescoat [a,*]

[a] *Inserm, U522, Rennes, F-35000 France; Univ Rennes 1, Rennes, F-35000 France; IFR 140, Rennes, F-35000 France*
[b] *UPRES 3891, Univ Rennes 1, Rennes, F-35000 France*
[c] *Novartis Institutes for BioMedical Research, Basel 4002, Switzerland*
[d] *CNRS, UMR 5616, Univ Grenoble, Grenoble, F-38000 France*

Received 21 February 2006; received in revised form 1 May 2006; accepted 1 May 2006
Available online 11 May 2006

Abstract

By comparing the antiproliferative effect of the iron chelators ICL670A and O-trensox in the human hepatoma cell line HUH7 and human hepatocyte cultures, we have shown that ICL670A decreased cell viability, inhibited DNA replication and induced DNA fragmentation more efficiently than O-trensox. O-trensox and ICL670A induced a cell cycle blockade in G0–G1 and S phases respectively. In parallel, ICL670A inhibited polyamine biosynthesis by decreasing ornithine decarboxylase and spermidine/spermine N_1-acetyltransferase activities. O-trensox increased polyamine biosynthesis and particularly putrescine level by stimulating spermidine–spermine N_1-acetyltransferase activity which could activate the polyamine retro-conversion pathway. Moreover, the two chelators exhibit some cytotoxic effect in the two culture models; ICL670A was more cytotoxic than O-trensox and higher concentrations of the two chelators were necessary to induce a cytotoxicity in primary cultures *versus* hepatoma cells. These results suggested that ICL670A has the most efficient antitumoral effect, blocks cell proliferation by a pathway different of O-trensox and may constitute a potential drug for anticancer therapy.

Keywords: Iron chelation; Human hepatocyte; Cell proliferation; Polyamine metabolism

1. Introduction

Iron plays a central role in the regulation of many cellular functions and is therefore an essential element for all living species. Dysregulation of its metabolism leads to an iron overload situation associated to cell and tissue deleterious effects or to an iron depletion leading to an inhibition of cell proliferation. Numerous iron chelators have been synthesised in order to treat primary or secondary iron overload diseases in hemochromatosis or thalassaemia respectively. In this context, they could prevent the development of hepatocellular carcinoma or inhibit the proliferation of tumor cells (Brissot and Loreal, 2002). The hyperproliferative effect of iron load has been shown in vitro on hepatocytes and in vivo on tumor cells (Thompson et al., 1991; Chenoufi et al., 1997). Moreover, the risk of developing a hepatocellular carcinoma appears to be related to the level and duration of iron overload (Deugnier and Turlin, 2001). On the other hand, iron depletion by chelators inhibits hepatocyte proliferation by inducing a cell cycle arrest which is frequently accompanied by an apoptosis-induced cell death (Chenoufi et al., 1995; Rakba et al., 2000). Among the different molecules synthesised, the hexadentate desferrioxamine (DFO) and the bidentate hydroxypyridinone deferiprone (CP20) are the major molecules used for the treatment of iron overload. However, DFO cannot be orally administered and clinical trials have shown that CP20 can induce a severe neutropenia (Porter and Davis, 2002). Then, chelators currently used for the therapy of iron overload diseases are not optimal compounds. It is therefore of great interest to develop new orally

* Corresponding author. Inserm U522, Hôpital Pontchaillou, 35033 Rennes cedex, France. Tel.: +33 2 99 54 74 06; fax: +33 2 99 54 01 37.
E-mail address: gerard.lescoat@rennes.inserm.fr (G. Lescoat).

0014-2999/$ - see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2006.05.001

efficient and non-toxic iron chelators. In this way, novel molecules such as O-trensox and the orally active ICL670A were synthesised and are of special interest since we have previously demonstrated that O-trensox inhibits cell proliferation and induces apoptosis with a higher efficiency than DFO in human hepatoma cell cultures (Caris et al., 1995; Rakba et al., 2000; Rouan et al., 2001).

Natural polyamines, putrescine spermidine and spermine, as iron, are necessary for cell proliferation. Their intracellular levels and uptake are significantly increased in tumor cell proliferation (Wallace et al., 2003). Recently, an interaction between the two metabolisms has been shown by involving the cell polyamine transport system as a potential cell entry pathway for iron in Chinese hamster ovary (CHO) cells (Gaboriau et al., 2004). Moreover, tumor promotion has been shown to be related to key enzymes of polyamine biosynthesis: ornithine decarboxylase (ODC) and polyamine retro-conversion pathway spermidine/spermine N_1-acetyltransferase (SSAT) (Yano et al., 1995; Bhasin et al., 2002). ODC, which converts ornithine in putrescine, has an increased activity in tumor cells. SSAT, which synthesises acetylated compounds from spermine and spermidine was shown to be activated in tumor cells.

In the present work, the antiproliferative effect of the two iron chelators, the hexadentate O-trensox and the tridentate ICL670A, has been compared in human hepatoma cell line HUH7 cultures and in primary human hepatocyte cultures. Cytotoxicity, DNA replication and cell cycle analysis have been correlated to intracellular polyamine levels. We show that the two iron chelators inhibit cell proliferation and induce cell death probably by an apoptosis pathway. This effect is correlated to a modulation of polyamine biosynthesis which results from an alteration of SSAT and/or ODC mRNA levels and polyamine intracellular concentrations. The comparison of the two molecules indicates that ICL670A has the most powerful antiproliferative potential.

2. Materials and methods

2.1. Human cell cultures

The human hepatoma cell line HUH7 was a gift from Dr. Ferry (Centre anticancéreux – Hôpital Ponchaillou – Rennes) and normal human hepatocytes for primary cultures were obtained after liver perfusion from the Centre de Ressources Biologiques (Hôpital Ponchaillou – Rennes). The two cell cultures were maintained in 75% minimum essential medium (MEM)–25% medium 199 (Hank's salts) supplemented with 10% of fetal calf serum, and containing, per ml, 7.5 IU penicillin – 50 μg streptomycin – 2 μmol glutamine – 0.05 μmol hydrocortisone hemisuccinate – 5 μg insulin – 1 mg serum albumin bovine. Proliferation of human hepatocytes in primary cultures was stimulated by adding 0.5 ng human recombinant Epidermal Growth Factor (EGF) per ml of culture medium 48 h after plating.

Chelators were added 72 h after plating HUH7 cells, and 48 h after plating human hepatocytes in primary cultures. Viability, proliferation, and polyamine metabolism of the cells were analysed 72 h after chelator exposures.

2.2. Chelator solutions

The hexadentate hydroxyquinoleine O-trensox (Caris et al., 1995), the tridentate hydroxyphenyltriazole ICL670A (Rouan et al., 2001) were compared. Concentrated solutions of each molecule were used as 5 mM in water for O-trensox, 5 mM in water–40% dimethyl sulfoxide (DMSO) for ICL670A. These solutions were sterilized by filtration on a 0.2 μm membrane. Chelators have been iron saturated with ferric iron ($FeCl_3$) in stoechiometric conditions of chelation, 1 mol of iron for 1 or 2 mol of O-trensox or ICL670A respectively.

Two controls have been done for each experiment: one with the standard culture medium, and the other with culture medium supplemented with DMSO at the same concentration than this used with ICL670A tests. DMSO supplemented controls didn't show any difference with respect to standard medium controls, therefore only the results obtained with controls without DMSO have been reported.

2.3. Cytotoxicity detection

Cytotoxicity was evaluated by the dosage of extracellular and intracellular lactate dehydrogenase activities (LDH, cytotoxicity detection kit – LDH, Roche, Penzberg, Germany) and mitochondrial succinate dehydrogenase activity (SDH) by the 3-(4,5-dimethylthiazol-2yl) 2,5-diphenyl tetrazolium bromide colorimetric assay (MTT, Sigma, St Louis, MO). Extracellular LDH activity was measured as described by the manufacturer, into cell culture medium and intracellular LDH activity was measured into cell lysates obtained by sonication of the cell layer in Phosphate-buffered saline (PBS) at low temperature. LDH activities were detected by reading absorbance at 485 nm. A LDH standard curve (0 to 1000 mU/ml) was used for the quantification of enzyme activities (L-lactate dehydrogenase, Sigma, St Louis, MO). Succinate dehydrogenase activity (SDH) was detected after 3 h incubation in 1 ml serum free medium containing 250 μg of MTT. After removing the supernatants, formazan salts were solubilized in DMSO and absorbance was red at 535 nm.

2.4. DNA fragmentation and cell cycle distribution

Cells were collected by trypsinisation and 1×10^6 cells were permeabilized with 0.5% PBS–saponine. After a RNAse treatment (100 μg/ml) (Sigma, St Louis, MO) the cells were stained with a Tris 10 mM–EDTA 1 mM solution containing 10 μg/ml of propidium iodide (Sigma, St Louis, MO). The DNA fragmentation and cell distribution in the different cycle phases were analysed by flow cytometry (FacsCalibur System, BD Biosciences, San Diego, USA). Analysis of the data was performed with ModFit software.

2.5. DNA replication

The last 24 h of the cell incubation with chelators, the cell culture medium was supplemented with 1 μCi/ml of [^{3}H]dimethylthymidine (Amersham, Les Ulis, France). DNA synthesis

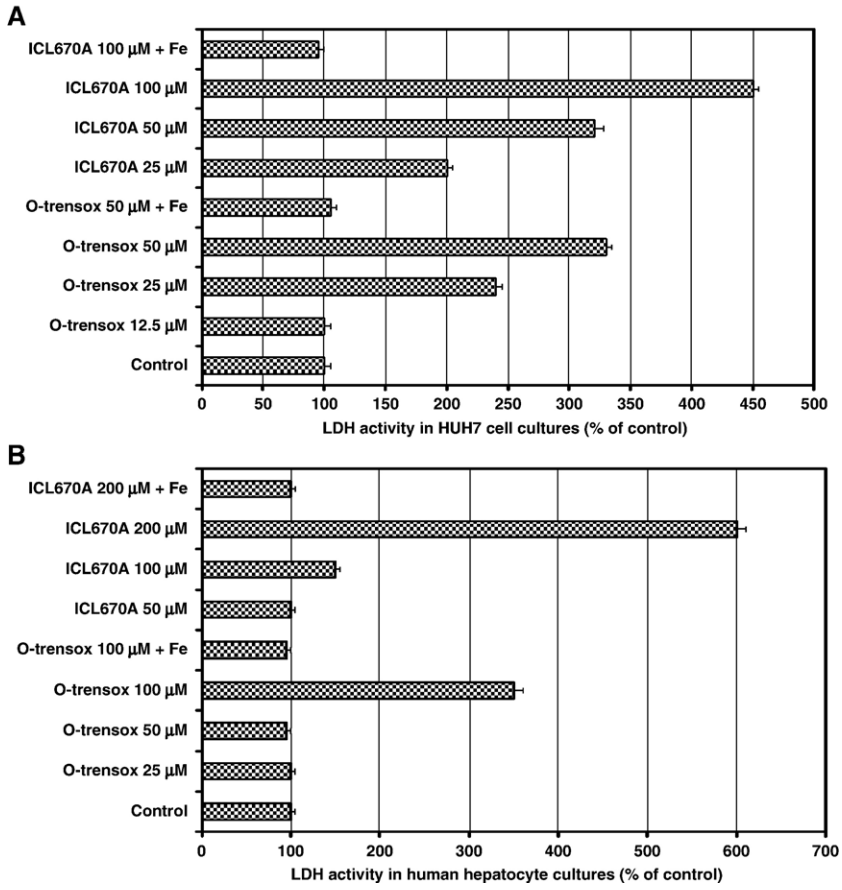

Fig. 1. Effect of the iron chelators O-trensox and ICL670A on extracellular LDH activity in the human hepatoma cell line HUH7 cultures (A) and human hepatocyte primary cultures (B). Cells were plated in 12 well plates at a density of 25,000 cells/well for HUH7 cells and 150,000 cells/well for human hepatocytes. 72 h after cell seeding, cells were treated during 72 h by the iron chelators at indicated concentrations. Extracellular LDH activity was reported to total LDH activity of the cultures. Data are means ± S.D. expressed in % of control of one representative of three independent experiments.

was evaluated by measuring thymidine incorporation into DNA collected after cell lysis by sonication in PBS. Results were expressed as a percentage of control value after quantification in cpm/mg of protein. Protein levels were measured according to a method adapted from the method of Bradford (Bio-Rad protein assay, Bio-Rad, Ivry sur Seine, France) and absorbance was red at 595 nm.

2.6. Polyamine levels

Putrescine, spermidine and spermine levels were measured as previously described by mass spectrometry analysis (Gaboriau et al., 2003). Cells were sonicated in perchloric acid 0.2 N. After an overnight incubation, the protein content was determined in the acid-insoluble material. The 1,10 diamino-dodecane (DAD, internal standard) was added to the supernatant which was treated with dansyl chloride (5 mg/ml) solubilized in acetone. Dansylated polyamines were extracted with cyclohexane, evaporated to dryness and resolubilized in acetonitrile. Mass spectrometry analysis was performed with 50 µl of acetonitrile polyamine solution. The polyamine levels were deduced from the ionic intensities of the selective ions and were corrected from that of DAD. Polyamine concentrations were deduced from a calibration curve of putrescine, spermidine and spermine (0 to 50 µM of each polyamine) and expressed with respect to the protein content.

2.7. RNA extraction and amplification

Total RNAs were extracted, from 25 cm^2 flasks at 80% of cell confluency, with SV total RNA isolation system (Promega Corporation, Madison, USA). After reverse transcription of total RNAs, ODC, SSAT and r18S transcripts were amplified by multiplex PCR by using three primers sets respectively: ODCfwd 5′-accggcgtaatcaacccagcg-3′, ODCrev 5′-tgcgtggtcatagagtatgc-3′; SSATfwd 5′-ttgggtcatggtgccagcctg-3′, SSATrev 5′-cacttctgcaaccaggcagtg-3′; 18Sfwd 5′-tcgaggcctgtaattggaa-3′, 18Srev 5′-cccaagatccaactacgagcttt (Eurogentec, Angers, France). Amplification fragments were obtained with the following PCR cycles: (94 °C–2 mn) – 40× (94 °C–30 s, 50 °C–40 s, 72 °C–30 s) – (72 °C–5 mn). The length of the co-amplified fragments of ODC (248 bp), SSAT (300 bp) and r18S (150 bp) were confirmed by electrophoresis through a 2.5% agarose gel in 0.5% Tris-acetate ethylene diamine tetracetic acid buffer. Density of the amplified fragments was evaluated by a DNA semi-quantification with DensiLab software.

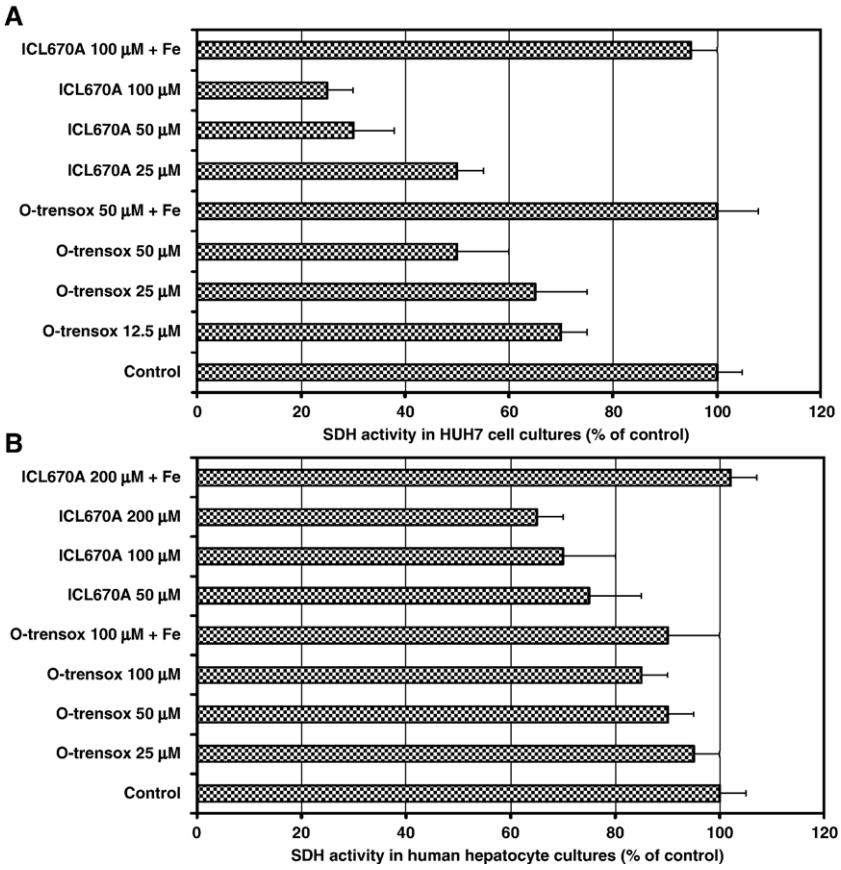

Fig. 2. Effect of the iron chelators O-trensox and ICL670A on succinate dehydrogenase activity — SDH – in the human hepatoma cell line HUH7 cultures (A) and human hepatocyte primary cultures (B). Cells were plated in 12 well plates at a density of 25,000 cells/well for HUH7 cells and 150,000 cells/well for human hepatocytes. 72 h after cell seeding, cells were treated during 72 h by the iron chelators at indicated concentrations. Mitochondrial succinate dehydrogenase activity was evaluated by the colorimetric MTT assay. Data are means ± S.D. expressed in % of control of one representative of three independent experiments.

2.8. Statistical analysis

Data are means ± S.D. from a representative experiment. All experiments have been repeated three times. The significance of the differences between means was determined by the non parametric Mann–Whitney test. Values of $p < 0.05$ were regarded as significant.

3. Results

3.1. Cytotoxicity of the iron chelators

The cytotoxic effects of O-trensox and ICL670A were compared in the two cell culture models using comparable iron-binding equivalent chelator concentrations. In HUH7 cell cultures, a dose-dependent increase in extracellular LDH activity was observed in the presence of O-trensox concentrations ranging from 12.5 to 50 µM or comparable ICL670A concentrations ranging from 25 to 100 µM (Fig. 1A, $p < 0.001$). In hepatocyte cultures, a significant cytotoxicity appeared only with 100 µM of O-trensox or ICL670A or 200 µM ICL670A (Fig. 1B, $p < 0.001$). In the two models, iron saturation of the chelators inhibits their cytotoxic effect (Fig. 1A, B). By comparing the results obtained in the two models with comparable iron-binding equivalent chelator concentrations, we observed that ICL670A exerts a higher cytotoxic effect than O-trensox. Moreover, higher concentrations of chelators were needed to induce cytotoxicity in primary cultures than in hepatoma cells (Fig. 1A, B).

3.2. Effect of iron chelators on mitochondrial succinate dehydrogenase

Modification of SDH activity can reflect either cytotoxicity or variation in cell proliferation and was compared in the two cell culture models using comparable iron-binding equivalent chelator concentrations. In HUH7 cell cultures, a dose-dependent decrease in SDH activity was observed in the presence of O-trensox concentrations ranging from 12.5 to 50 µM or comparable ICL670A concentrations ranging from 25 to 100 µM (Fig. 2A, $p < 0.001$). In hepatocyte cultures, a progressive slight dose-dependent decrease in SDH activity was observed in the presence of O-trensox concentrations ranging from 25 to 100 µM (Fig. 2B, $p < 0.001$) or comparable ICL670A concentrations ranging from 50 to 200 µM (Fig. 2B, $p < 0.001$). In the two models, iron saturation of the chelators inhibits their effect (Fig. 2A, B). By comparing the results obtained in the two models with comparable iron-binding equivalent chelator concentrations, we observed that ICL670A exerts a higher effect than O-trensox. Moreover, higher concentrations of chelators were necessary to induce an SDH activity decrease in primary cultures compared to hepatoma cells (Fig. 2A, B).

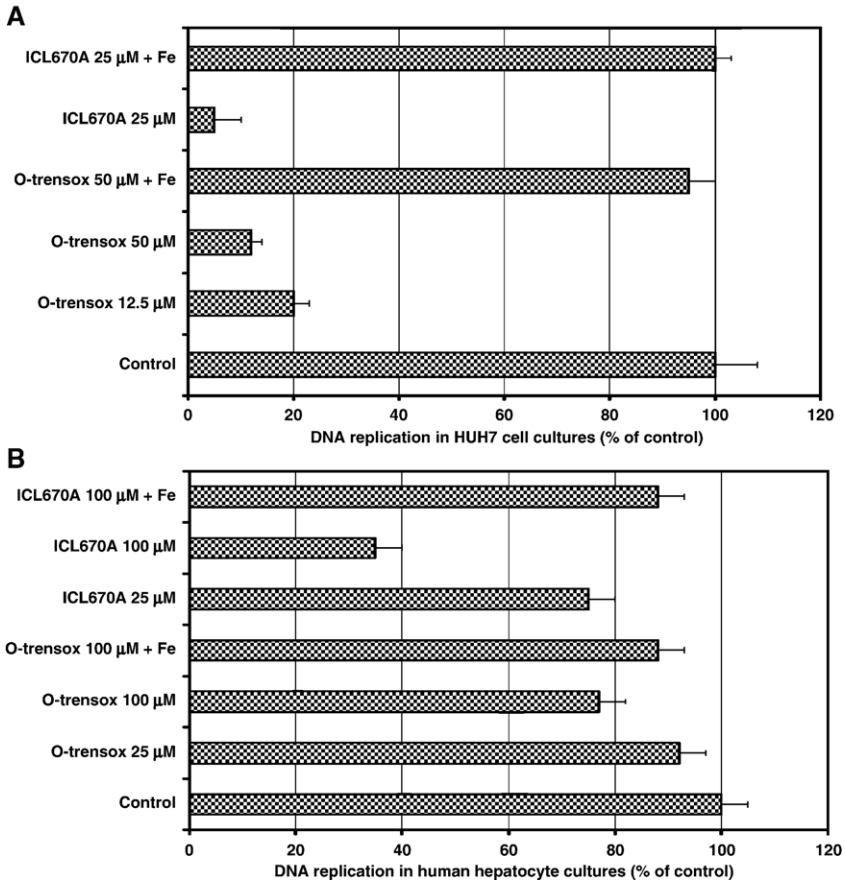

Fig. 3. Effect of the iron chelators O-trensox and ICL670A on DNA replication in human hepatoma cell line HUH7 cultures (A) and human hepatocyte primary cultures (B). Cells were plated on 12 well plates. After 48 h of exposure to the chelators, the cells were further grown 24 h with [H^3]thymidine and then sonicated in PBS. Incorporation of [H^3] thymidine was performed on cell lysate and expressed as cpm/mg protein. Data are means ± S.D. expressed in % of control of one representative of three independent experiments.

3.3. Effect of iron chelators on DNA replication

In the HUH7 cell cultures, a decrease in DNA replication (80 up to 88%) was observed in the presence of 12.5 or 50 µM O-trensox ; a higher decrease (95%) was obtained with 25 µM ICL670A (Fig. 3A, $p<0.001$). In hepatocyte cultures, a dose-dependent decrease in DNA replication was observed in the presence of 25 and 100 µM of O-trensox (8 up to 23%) or similar ICL670A concentrations (25 up to 65%) (Fig. 3B, $p<0.001$). In the two models, iron saturation of the chelators

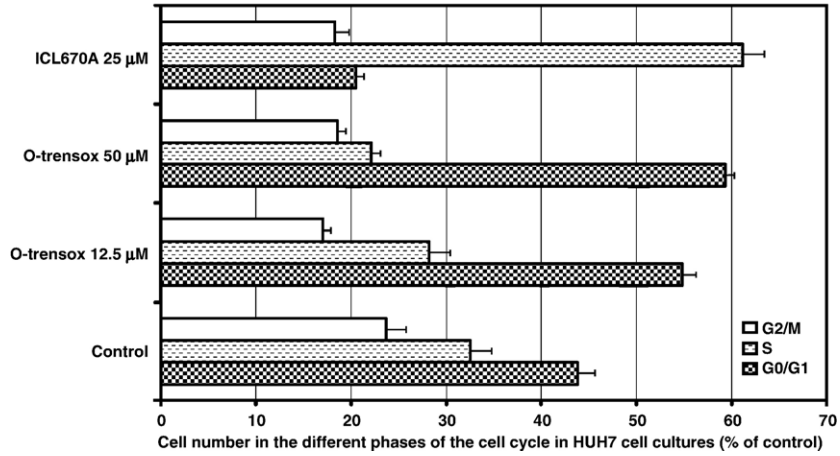

Fig. 4. Effect of the iron chelators O-trensox and ICL670A on cell cycle progression of human hepatoma cell line HUH7 cultures. After 72 h of incubation with the chelators, cells were collected by brief trypsinisation and cell membranes were permeabilized by PBS–saponine 0.5%. Cells were treated by RNAse and DNA was stained with propidium iodide. Cell DNA content was analysed by flow cytometry and quantification was performed with ModFit software. Data are means ± S.D. of one representative of three independent experiments.

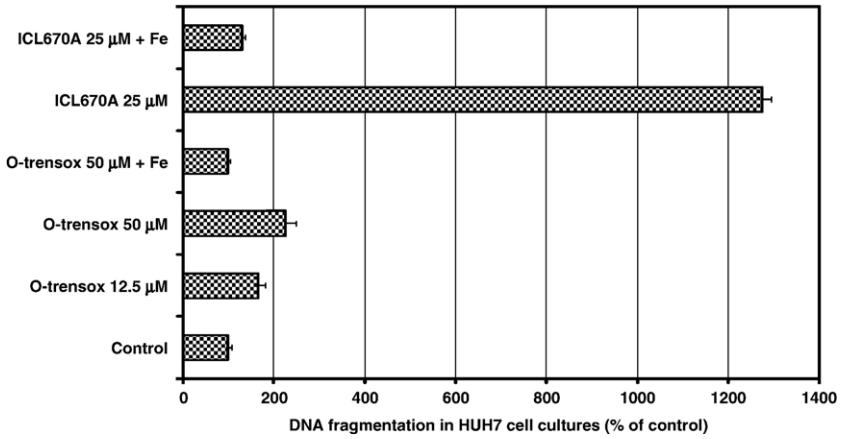

Fig. 5. Effect of the iron chelators O-trensox and ICL670A on nuclear DNA fragmentation in human hepatoma cell line HUH7 cultures. After 72 h of incubation with the chelators, cells were collected by brief trypsinisation and cell membranes were permeabilized by PBS–saponine 0.5%. Cells were treated by RNAse and DNA was stained with propidium iodide. DNA content of cells was analysed by flow cytometry and quantification was performed with ModFit software. Data are means ± S.D. expressed in % of control of one representative of three independent experiments.

inhibited their antiproliferative effect (Fig. 3A, B). We noticed also that higher chelator concentrations were necessary to inhibit cell proliferation in primary cultures and that ICL670A was more cytostatic than O-trensox (Fig. 3A, B).

3.4. Effect of iron chelators on DNA content and cell cycle distribution

We observed that higher iron chelator concentrations are necessary to inhibit cell proliferation in primary cultures compared to HUH7 hepatoma cells. Moreover, human hepatocyte cultures stimulated by EGF were able to proliferate but dead by apoptosis after one cell cycle. Therefore, this type of cell culture did not appear to be the more appropriate model for our cell cycle study and polyamine metabolism. Therefore, these two analyses have been performed only in HUH7 cells. We observed that 12.5 or 50 µM O-trensox induced an accumulation of HUH7 cells in G0–G1 phase of the cell cycle (57% *versus* 44%, Fig. 4, $p<0.001$). A blockade of HUH7 cells in S phase of the cell cycle was observed with 25 µM ICL670A (61% *versus* 32%, Fig. 4, $p<0.001$).

3.5. Effect of iron chelators on DNA fragmentation

Flow cytometry analysis of HUH7 cells stained by propidium iodide after treatment with the two iron chelators (12.5 or 50 µM O-trensox and 25 µM ICL670A) showed a large increase of fragmented DNA (Fig. 5, $p<0.001$). In the same conditions of iron-binding equivalents, the most significant DNA alteration was observed with 25 µM ICL670A compared to 12.5 µM O-trensox. Iron saturation of the chelators, inhibited significantly DNA fragmentation (Fig. 5, $p<0.001$).

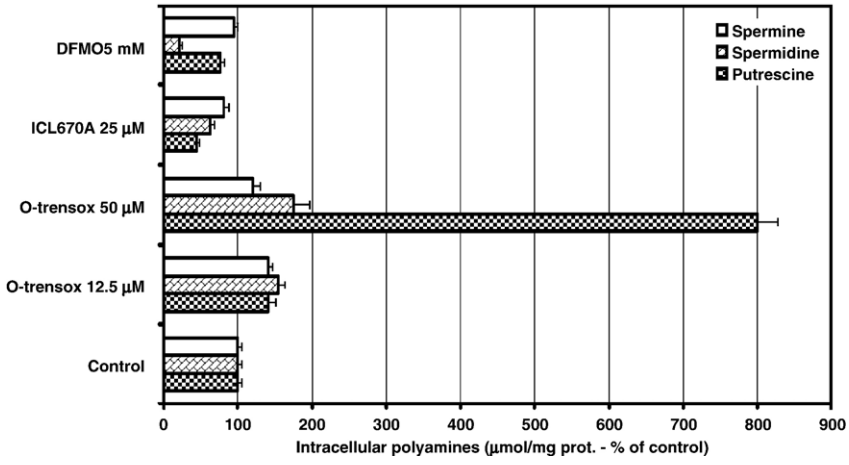

Fig. 6. Effect of the iron chelators O-trensox and ICL670A on intracellular levels of putrescine, spermidine and spermine in human hepatoma cell line HUH7 cultures. After 72 h of incubation with the chelators, cells were collected by brief trypsinisation and sonicated in 0.2 N perchloric acid. After overnight incubation at 4 °C, cell lysates were pelleted and the supernatant was dansylated. Dansylated polyamines were extracted as described in the Materials and methods section and analysed by mass spectrometry. Polyamine concentrations were corrected from the protein content of the cultures. Data are means ± S.D. expressed in % of polyamine amount of control of one representative of three independent experiments.

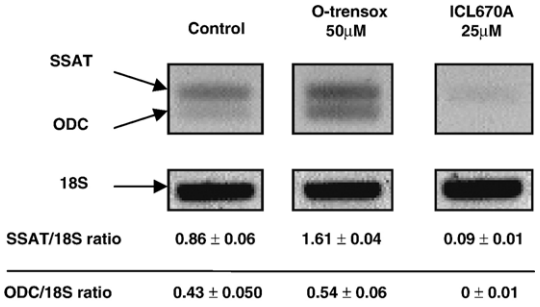

Fig. 7. Effect of the iron chelators O-trensox and ICL670A on ODC and SSAT mRNA levels in human hepatoma cell line HUH7 cultures. Total RNAs were purified from HUH7 cells after 72 h of incubation with the chelators. After a reverse transcription, multiplex PCR amplification of ODC, SSAT and r18S transcripts were performed and analysed through a 2.5% agarose gel in 0.5% TAE buffer. Density of the three amplified fragments was obtained by DensiLab software. Data are means±S.D. of one representative of two independent repeated experiments.

3.6. Effect of iron chelators on intracellular polyamine levels

In HUH7 cells, 12.5 μM O-trensox increased putrescine, spermidine and spermine intracellular concentrations and the putrescine level was largely increased by 50 μM O-trensox (Fig. 6, $p<0.001$). In parallel, HUH7 cells showed a high increase of SSAT mRNA level and to a lesser extent of ODC mRNA level (Fig. 7). ICL670A had an opposite effect by decreasing the levels of the three polyamines (Fig. 6, $p<0.05$ or 0.01)). A comparable depletion of putrescine and spermidine was obtained in the human hepatoma cell line using DL-alpha-difluoromethylornithine — DFMO — (Fig. 6). In accordance with these results, a significant decrease of SSAT and ODC mRNA levels was observed in cells treated with ICL670A (Fig. 7, $p<0.001$).

4. Discussion

O-trensox and ICL670A have been shown to mobilize iron of iron-loaded hepatocytes and consequently to induce cellular protection against iron damage (Rakba et al., 1998; Hershko et al., 2001). This intracellular iron depletion was correlated to a cytotoxicity of O-trensox in human hepatoma cell lines (Rakba et al., 2000). In the present study, in HUH7 cells and hepatocyte cultures, the higher cytotoxicity, revealed by an increase in extracellular LDH activity, was obtained with ICL670A compared to O-trensox. O-trensox induced the same toxicity as observed in a previous work in HepG2 cells (Rakba et al., 2000). We observed also, in HUH7 cells and primary human hepatocyte cultures, an inhibition of the cytotoxic effect by iron saturation of the chelators. Moreover, higher concentrations of the chelators were necessary to induce cytotoxicity in primary hepatocyte cultures than in hepatoma cells. This observation is in agreement with previous results indicating that the iron chelator deferiprone was toxic for rat hepatoma cells (at concentrations near 50 μM) whereas it did not alter the viability of rat hepatocytes in primary cultures (Kicic et al., 2001).

Modification of SDH activity can reflect either cytotoxicity or variation in cell proliferation. A decrease of SDH activity corroborating an extracellular LDH increase was reported in HepG2 cells exposed to CP20 (Chenoufi et al., 1998; Kicic et al., 2001). In our study, we made the same observation with the two tested iron chelators, O-trensox and ICL670A. In HUH7 cells and primary hepatocyte cell cultures, ICL670A induced the highest decrease of SDH activity, but also the higher increase in extracellular LDH activity. By analysing the results obtained in the two models using comparable iron-binding equivalent chelator concentrations, we demonstrated that ICL670A decreased more SDH activity than O-trensox. The chelator effect did not persist longer in the condition of iron saturation and higher chelator concentrations were also necessary to decrease SDH activity in primary cultures compared to hepatoma cells.

The antiproliferative ability of iron chelators, like O-trensox and CP20, has been previously shown in different hepatoma cell lines (Chenoufi et al., 1998; Rakba et al., 2000; Kicic et al., 2001). In HUH7 cells, and to a lesser extent in hepatocyte cultures, the chelators O-trensox and ICL670A induced an inhibition of DNA replication which was closely correlated to the decrease of mitochondrial SDH activity. However, in the hepatoma cell line, the comparison of the two chelators in similar iron-binding equivalent conditions indicated a higher inhibition of DNA replication with ICL670A than with O-trensox. This result was confirmed in primary human hepatocyte cultures using similar concentrations of O-trensox or ICL670A. Iron saturation of the two molecules prevented nearly completely the decrease of DNA synthesis. Moreover, higher chelator concentrations were necessary to inhibit cell proliferation in the primary cultures *versus* hepatoma cells and ICL670A was more cytostatic than O-trensox.

O-trensox and CP20 have been shown to arrest HepG2 cells in G1 phase and in S phase of the cell cycle respectively (Chenoufi et al., 1998; Rakba et al., 2000). In our study, 12.5 μM O-trensox induced an accumulation of HUH7 cells in G0–G1 phase of the cell cycle; this result was confirmed and amplified using the higher concentration 50 μM. A comparable iron-binding equivalent concentration of ICL670A (25 μM) induced a blockade of HUH7 cells in S phase of the cell cycle.

The cytotoxic and cytostatic properties of O-trensox and CP20 have been already correlated to DNA fragmentation in human tumor cells, which reflected cell death by an apoptosis pathway (Rakba et al., 2000; Yasumoto et al., 2004). Here, the flow cytometry analysis of HUH7 cells stained by propidium iodide after treatment with the iron chelators O-trensox and ICL670A showed a large increase of fragmented DNA. In similar conditions of iron-binding equivalents, the most significant DNA alteration was observed with ICL670A compared to O-trensox. The previous data reported with O-trensox (Rakba et al., 2000) indicated that the DNA fragmentation observed reflected a cell death by apoptosis since it was correlated to an increase of caspase 3 activity. Thus, in accordance to LDH and SDH activities and to DNA fragmentation measurements, the cell damages observed with ICL670A could also be related to apoptosis-induced cell death. This conclusion was reinforced by a recent result showing an increase of caspase 3 activity in rat hepatoma cell line FAO cultures treated by ICL670A (unpublished observation).

The intracellular polyamine levels and the enzymes of their metabolism have been shown to be differentially regulated

during cell cycle progression and in the induction of cell death apoptosis (Schipper et al., 2000; Oredsson, 2003). ODC and SSAT activities were shown to be high in tumor and proliferating cells stimulated by growth factors (Matsui and Pegg, 1980). On the other hand, inhibition of the first rate-limiting enzyme of polyamine biosynthesis, ornithine decarboxylase (ODC), by DL-α-difluoromethylornithine (DFMO) induced a cell cycle blockade in G1 or G2 cycle phases of numerous cell lines (Marty et al., 2000). In the same way, induction of spermidine/spermine N_1-acetyltransferase (SSAT) was correlated to growth inhibition (Ichimura et al., 1998). In our study, putrescine level was largely increased in HUH7 cells treated by O-trensox. In parallel, we observed also an increase of SSAT mRNA level as previously reported with the growth arrest of HeLa cells (Ichimura et al., 1998). Moreover, this high putrescine intracellular level could also be correlated to the slight increase of ODC mRNA level. ICL670A had an opposite effect by decreasing the levels of the three polyamines. A comparable depletion of polyamines was obtained by treating the human hepatoma cell line with DFMO. In accordance with these results, a significant decrease of ODC and SSAT mRNA levels was observed in cells treated with ICL670A. So, O-trensox and ICL670A block cell proliferation which is followed by apoptosis; however, ICL670A inhibits cell proliferation and exerts an apoptotic effect with a higher efficiency than O-trensox. The higher antiproliferative and apoptotic effect of ICL670A may be explained by two observations: the decrease of putrescine and SSAT mRNA levels. Indeed, previous results suggested that putrescine is essential for the cell to enter S-phase, possibly pushing the cell through the G_1 restriction point prior to DNA synthesis (Wallace et al., 2003). Concerning SSAT expression, recent results, obtained in different cancer cell lines, have demonstrated that the upregulation of SSAT mRNA, in an iron deficient condition using DFO, may exert a protective function in apoptotic cell death (Kim et al., 2005). So, the downregulation of SSAT mRNA level induced by ICL670A may also contribute to its higher antiproliferative and apoptotic effect compared to O-trensox.

In conclusion, we demonstrated that the iron chelators O-trensox and ICL670A inhibit the proliferation of HUH7 human hepatoma cells and human hepatocytes. Each of them induced growth inhibition by a blockade at different phases of the cell cycle associated to a deregulation of polyamine metabolism. O-trensox induces a cell cycle arrest in G0–G1 of the cell cycle; this blockade is associated to a large increase of putrescine which could result mainly from the activation of SSAT, the key enzyme involved in the retro-conversion pathway of polyamine metabolism. The recent orally active chelator ICL670A appears to be the most powerful agent. It induces a cell cycle arrest in S phase associated with a decrease of the polyamine levels which could result from an inhibition of polyamine biosynthesis probably by ODC inactivation. Moreover, an important point is also that the cytotoxic, the cytostatic and the apoptotic effect of ICL670A observed in the hepatoma cells appeared with less efficiency in normal hepatocytes. This study gives indications of a strong relation between iron depletion and polyamine metabolism which appeared to be dependent of the chemical structure of the iron chelator which is probably closely correlated to biological properties independent of iron depletion. Since both iron and polyamines are known to play an essential role in controlling cell proliferation, the present results indicate that ICL670A, by inhibiting SSAT expression, may constitute a promising therapeutic molecule in the strategy for cancer treatment.

Acknowledgments

This work was supported by the “5th PCRDT European contract No. QLRT-2001-00444: iron in hemochromatosis: deleterious effects of an essential nutrient” and ACI “CAPFER: nouveaux outils chimiques et analytiques pour la détection et le dosage du fer ; application à l’étude de son métabolisme ; no. 03 2 135”. We thank Dr O. Loréal for the discussion about this work.

References

Bhasin, G., Kauser, H., Athar, M., 2002. Low iron state is associated with reduced tumor promotion in a two-stage mouse skin carcinogenesis model. Food Chem. Toxicol. 40, 1105–1111.

Brissot, P., Loreal, O., 2002. Role of non-transferrin-bound iron in the pathogenesis of iron overload and toxicity. Adv. Exp. Med. Biol. 509, 45–53.

Caris, C., Baret, P., Beguin, C., Serratrice, G., Pierre, J.L., Laulhere, J.P., 1995. Metabolization of iron by plant cells using O-Trensox, a high-affinity abiotic iron-chelating agent. Biochem. J. 312 (Pt 3), 879–885.

Chenoufi, N., Hubert, N., Loréal, O., Morel, I., Pasdeloup, N., Cillard, J., Brissot, P., Lescoat, G., 1995. Inhibition of iron toxicity in rat and human hepatocyte cultures by the hydroxypyridin-4-ones CP20 and CP94. J. Hepatol. 23, 166–173.

Chenoufi, N., Loréal, O., Drénou, B., Cariou, S., Hubert, N., Leroyer, P., Brissot, P., Lescoat, G., 1997. Iron may induce DNA synthesis and repair in rat hepatocytes stimulated by EGF/pyruvate. J. Hepatol. 26, 650–658.

Chenoufi, N., Drénou, B., Loréal, O., Pigeon, C., Brissot, P., Lescoat, G., 1998. Antiproliferative effect of deferiprone on the HepG2 cell line. Biochem. Pharmacol. 56, 431–437.

Deugnier, Y., Turlin, B., 2001. Iron and hepatocellular carcinoma. J. Gastroenterol. Hepatol. 16, 491–494.

Gaboriau, F., Havouis, R., Moulinoux, J.P., Delcros, J.G., 2003. Atmospheric pressure chemical ionization-mass spectrometry method to improve the determination of dansylated polyamines. Anal. Biochem. 318, 212–220.

Gaboriau, F., Kreder, A., Clavreul, N., Moulinoux, J., Delcros, J.G., Lescoat, G., 2004. Polyamine modulation of iron uptake in CHO cells. Biochem. Pharmacol. 67, 1629–1637.

Hershko, C., Konijn, A.M., Nick, H.P., Breuer, W., Cabantchik, Z.I., Link, G., 2001. ICL670A: a new synthetic oral chelator: evaluation in hypertransfused rats with selective radioiron probes of hepatocellular and reticuloendothelial iron stores and in iron-loaded rat heart cells in culture. Blood 97, 1115–1122.

Ichimura, S., Hamana, K., Nenoi, M., 1998. Significant increases in the steady states of putrescine and spermidine/spermine N1-acetyltransferase mRNA in HeLa cells accompanied by growth arrest. Biochem. Biophys. Res. Commun. 243, 518–521.

Kicic, A., Chua, A.C., Baker, E., 2001. Effect of iron chelators on proliferation and iron uptake in hepatoma cells. Cancer 92, 3093–3110.

Kim, K., Ryu, J.H., Park, J.W., Kim, M.S., Chun, Y.S., 2005. Induction of a SSAT isoform in response to hypoxia or iron deficiency and its protective effects on cell death. Biochem. Biophys. Res. Commun. 331, 78–85.

Marty, C., Mori, G., Sabini, L., Rivarola, V., 2000. Effects of alpha-difluoromethylornithine on the cyclin A expression in Hep-2 cells. Biocell 24, 49–52.

Matsui, I., Pegg, A.E., 1980. Effect of thioacetamide, growth hormone or partial hepatectomy on spermidine acetylase activity of rat liver cytosol. Biochim. Biophys. Acta 633, 87–94.

Oredsson, S.M., 2003. Polyamine dependence of normal cell-cycle progression. Biochem. Soc. Trans. 31, 366–370.

Porter, J.B., Davis, B.A., 2002. Monitoring chelation therapy to achieve optimal outcome in the treatment of thalassaemia. Best Pract. Res. Clin. Haematol. 15, 329–368.

Rakba, N., Aouad, F., Henry, C., Caris, C., Morel, I., Baret, P., Pierre, J.L., Brissot, P., Ward, R.J., Lescoat, G., Crichton, R.R., 1998. Iron mobilisation and cellular protection by a new synthetic chelator O-Trensox. Biochem. Pharmacol. 55, 1797–1806.

Rakba, N., Loyer, P., Gilot, D., Delcros, J.G., Glaise, D., Baret, P., Pierre, J.L., Brissot, P., Lescoat, G., 2000. Antiproliferative and apoptotic effects of O-Trensox, a new synthetic iron chelator, on differentiated human hepatoma cell lines. Carcinogenesis 21, 943–951.

Rouan, M.C., Marfil, F., Mangoni, P., Sechaud, R., Humbert, H., Maurer, G., 2001. Determination of a new oral iron chelator, ICL670, and its iron complex in plasma by high-performance liquid chromatography and ultraviolet detection. J. Chromatogr., B, Biomed. Sci. Appl. 755, 203–213.

Schipper, R.G., Penning, L.C., Verhofstad, A.A., 2000. Involvement of polyamines in apoptosis. Facts and controversies: effectors or protectors? Semin. Cancer Biol. 10, 55–68.

Thompson, H.J., Kennedy, K., Witt, M., Juzefyk, J., 1991. Effect of dietary iron deficiency or excess on the induction of mammary carcinogenenis by 1-methyl-1-nitrosourea. Carcinogenesis 12, 111–114.

Wallace, H.M., Fraser, A.V., Hughes, A., 2003. A perspective of polyamine metabolism. Biochem. J. 376, 1–14.

Yano, T., Obata, Y., Yano, Y., Otani, S., Ichikawa, T., 1995. Stimulating effect of excess iron feeding on spontaneous lung tumor promotion in mice. Int. J. Vitam. Nutr. Res. 65, 127–131.

Yasumoto, E., Nakano, K., Nakayachi, T., Morshed, S.R., Hashimoto, K., Kikuchi, H., Nishikawa, H., Kawase, M., Sakagami, H., 2004. Cytotoxic activity of deferiprone, maltol and related hydroxyketones against human tumor cell lines. Anticancer Res. 24, 755–762.