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Antiproliferative Effect of Deferiprone on the Hep G2 Cell Line

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ABSTRACT. Iron is an essential element in cellular metabolism and the growth of all living species, and is involved in DNA replication. The risk of hepatocellular carcinoma development is associated with an increase in iron availability. The aim of the present work was to investigate the effect of an oral iron chelator, deferiprone (CP20), on HepG2 cell-line proliferation in culture. HepG2 cell cultures were maintained in the absence of fetal calf serum (FCS) and in the presence or not (control cultures) of CP20 at the concentrations of 50 or 100 μM; deferoxamine (DFO) was used as an iron chelator reference. Cell proliferation was investigated by the analysis of DNA synthesis using [^{3}H] methyl-thymidine incorporation and of the cell cycle by flow cytometry. Iron chelation efficiency in the culture model was studied by analyzing the effect of CP20 on radioactive iron uptake, intracellular ferritin level, and transferrin receptor expression. CP20, at the concentration of 50 or 100 μM, inhibited DNA synthesis after 48 hr of incubation and induced an accumulation of the cells in the S phase of the cell cycle. Iron chelators inhibited cellular iron uptake, decreased intracellular ferritin level, and increased transferrin receptor protein and mRNA levels. Our results show that CP20 as well as deferoxamine inhibit HepG2 cell proliferation and block cell cycle in the S phase. BIOCHEM PHARMACOL **56**;4:431–437, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. deferoxamine; deferiprone; iron; DNA synthesis; cell cycle; HepG2 cell line

The hypothesis that iron is implicated in the development of tumor cell growth is supported by several studies using different models [1–2]. Moreover, HCC‖ is a frequent complication of genetic hemochromatosis [3–5]. The risk of developing HCC seems to be related to both the rate and duration of iron overload [6, 7]. In addition, the occurrence of HCC in hemochromatotic patients without cirrhosis has been reported [8]. Furthermore, in non-hemochromatotic patients who develop HCC during other liver diseases, it has recently been reported that liver iron concentration in nontumoral areas is higher than in patients without HCC [9].

All cells require iron, and neoplastic cells have a high iron requirement related to their rapid rate of replication [10, 11]. In the absence of iron, cells are unable to progress in the cell cycle [12, 13]. Therefore, iron appears to be a regulatory factor of the cell cycle. Previous studies have shown that DFO, a strong and specific iron chelator of bacterial origin, is able to inhibit DNA synthesis and tumor cell proliferation in a number of hepatoma [14] and neuroblastoma [15] cell lines. Consequently, DFO has been proposed for the treatment of neuroblastoma in association with chemotherapy multi-agents [16]. However, DFO is poorly absorbed by the gastrointestinal tract. Various new iron chelators have been designed for clinical use [17]. Among them, CP20 has been proposed as an oral iron chelator for the treatment of secondary iron overload [18, 19]. The aim of this work was to investigate, in the HepG2 cell line, the potential effect of CP20 as an antiproliferative agent, and the effect of iron depletion on the cell cycle.

MATERIALS AND METHODS

Cell Culture

The human hepatoblastoma cell line HepG2 used in this study was obtained by Knowles *et al.* [20] and maintained in a medium containing 75% minimum essential medium and 25% medium 199, supplemented with 10% FCS and containing per mL: penicillin (7.5 IU), bovine insulin (5 μg), BSA (1 mg), streptomycin (50 μg), $NaHCO_3$ (2.2 mg) and 7×10^{-7} M hydrocortisone hemisuccinate. For the experiments, cells were maintained in the same medium as above but deprived of FCS, and treated or not by DFO (50 or 100 μM) or CP20 (50 or 100 μM) for 24 or 48 hr.

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‖ *Abbreviations:* CP20, deferiprone; DFO, deferoxamine; HCC, hepatocellular carcinoma; LDH, lactate dehydrogenase; MTT, (3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide); and TCA, trichloroacetic acid.

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DNA Synthesis Measurement

[^{3}H]methyl-thymidine (Amersham) was added to the culture medium at a final concentration of 1 μCi/mL for 24 hr, before cell harvesting. DNA synthesis was evaluated by measuring [^{3}H]methyl-thymidine incorporation into TCA-precipitable DNA in presence of iron chelators (50, 100 μM), or in presence of iron chelators and hydroxyurea (5 mM) for 24 hr. Results were expressed as percent of control values.

LDH Assay

LDH activity was measured in the culture medium using LDH Kit (Bayer Diagnostics) adapted to the Alcyon automatic analyzer. Results were expressed as percent of control values.

MTT Assay

MTT assay was performed according to the method of Twentyman *et al.* [21]. This technique is based on the reduction of the yellow tetrazolium salt to the blue formazan product by mitochondrial dehydrogenases of living cells. After MTT addition (0.5 mg/mL), the plates were covered and returned to the 37° incubator for 2 hr, the optimal time for formazan product formation. Following incubation, the supernatant was removed from the wells, and the formazan product was dissolved in 100 μL of DMSO. The 96-well plates were read on a Titertek Multiskan plate reader at an absorption wavelength of 540 nm. Results were expressed as percent of control values.

Flow-Cytometric Analysis

After cell trypsinization, DNA content was measured in cells using a DNA prep kit (Coulter). Human lymphocytes were used as standard. Cell-cycle analysis was performed using a flow cytometer (Cytoron) equipped with an argon-ion laser and the M-cycle software system (Phoenix Flow Systems).

RNA Extraction and Northern Blot Analysis

Cells were scraped in denaturing solution and total RNA was prepared as previously described [22], dissolved in sterile water, and stored at −80°. For Northern blotting, 10 μg of each RNA sample were resolved by electrophoresis in denaturing 6% formaldehyde 1% agarose gel, and transferred onto Hybond-N+ nylon filters (Amersham) in 20× standard saline citrate (3 M NaCl , 0.3 M trisodium citrate) buffer. Filters were then hybridized with (α^{32}-P) human transferrin receptor cDNA probe labeled using a rediprime DNA labeling system (Amersham). The transferrin receptor cDNA probe was prepared from the plasmid pcDTR1, obtained from the American Type Culture Collection, by excision of the 4.9-Kb insert with BamH1 restriction enzyme. Filters were autoradiographed at −80° and hybridization signals were quantified by densitometry and integration of unsaturated signals.

Iron Uptake

Cell cultures were maintained in the presence of 1 μM ^{55}Fe-citrate (1 mCi/mg of Fe; Amersham) for 24 hr. Iron uptake by the cell layer was analyzed after 1, 3 and 24 hr of incubation. Cell cultures were then rinsed and sonicated in 1 mL of PBS, and radioactive incorporation was determined by scintillation counting. Results were expressed as percent of control values.

Ferritin Assay

Intracellular concentrations of ferritin were measured by a ferritin ^{125}I radioimmunometric method using the magic® ferritin RIA kit (Corning Magnetic Immunoindustries, Ciba Corning Diagnostics). Intracellular ferritin levels were expressed as percent of the control values.

Transferrin Receptor Expression

CD71 antibody recognizes cell surface antigens corresponding to the transferrin receptors. The quantification of the number of binding sites was performed by flow cytometry with an indirect immunofluorescence using CD71 monoclonal antibody (Immunotech) and Qifikit reagents (DAKO) according to the manufacturers' instructions. In brief, internal standard beads were used to relate the mean fluorescence intensity, calculated from flow cytometry histograms, to the mean number of antibody molecules bound per cell.

Statistical Analysis

Results were expressed as means ± SEM of three independent experiments in which each experimental condition was carried out in triplicate. Statistical analysis was performed using the Student's *t*-test. The significant level was set at 0.05.

RESULTS

DNA Synthesis and Cell-Cycle Analysis

We investigated the effect of 50 and 100 μM DFO or CP20 on DNA synthesis and proliferation in the HepG2 cell line. No significant effect of 50 or 100 μM CP20 and DFO on DNA synthesis was observed after 24 hr of exposure (Fig. 1). However, a dose-dependent decrease was observed after 48 hr of treatment with iron chelators (DFO 50 μM, $P < 0.05$; DFO 100 μM, $P < 0.01$; CP20 50 μM, $P < 0.05$; CP20 100 μM, $P < 0.01$; Fig. 1). The decrease in DNA synthesis, as compared to cultures treated with hydroxyurea alone, was important when iron chelators were added in the presence of 5 mM hydroxyurea (DFO 50 or 100 μM, $P <$

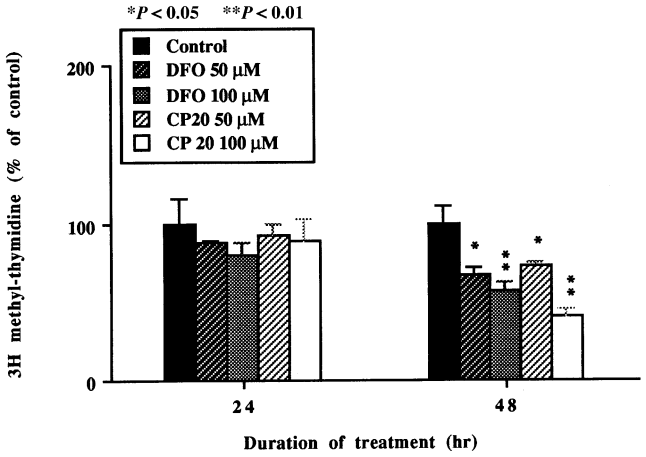

FIG. 1. Measurement of [^{3}H] methyl-thymidine incorporation into DNA in HepG2 cell line control cultures or cultures maintained for 24 and 48 hr in the presence of DFO or CP20 (50 or 100 μM). Addition of 1 μCi/mL of [^{3}H] methyl-thymidine was performed during the last 24 hr before cell harvesting.

0.01; CP20 50 or 100 μM, $P < 0.01$; Fig. 2). However, no dose-dependent effect was observed. In addition, analysis of the cell cycle after 48 hr of exposure to the iron chelators showed a decreased percentage of cells in the G0-G1 phase (DFO 50 μM, $P < 0.01$; CP20 100 μM, $P < 0.01$), but a significant increase of the percentage of cells in the S phase (DFO 50 or 100 μM, $P < 0.01$; CP20 50 or 100 μM, $P < 0.01$). The percentage of cells in the G2-M phase was significantly decreased only in the presence of DFO (DFO 50 μM, $P < 0.01$; DFO 100 μM, $P < 0.05$), while CP20 was without significant effect (Fig. 3, Table 1).

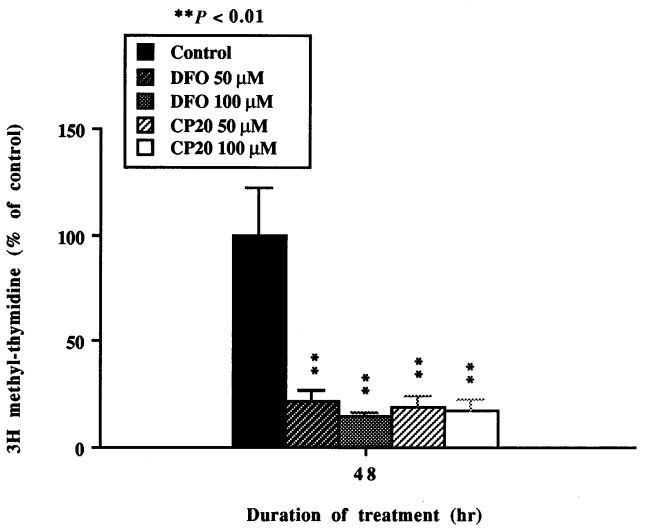

FIG. 2. Measurement of [^{3}H] methyl-thymidine incorporation into DNA in HepG2 cells cultured in the presence of hydroxyurea. Addition of 1 μCi/mL of [^{3}H] methyl thymidine and 5 mM hydroxyurea was performed during the last 24 hr before cell harvesting in control cultures and in cultures maintained for 48 hr in the presence of DFO or CP20 (50 or 100 μM).

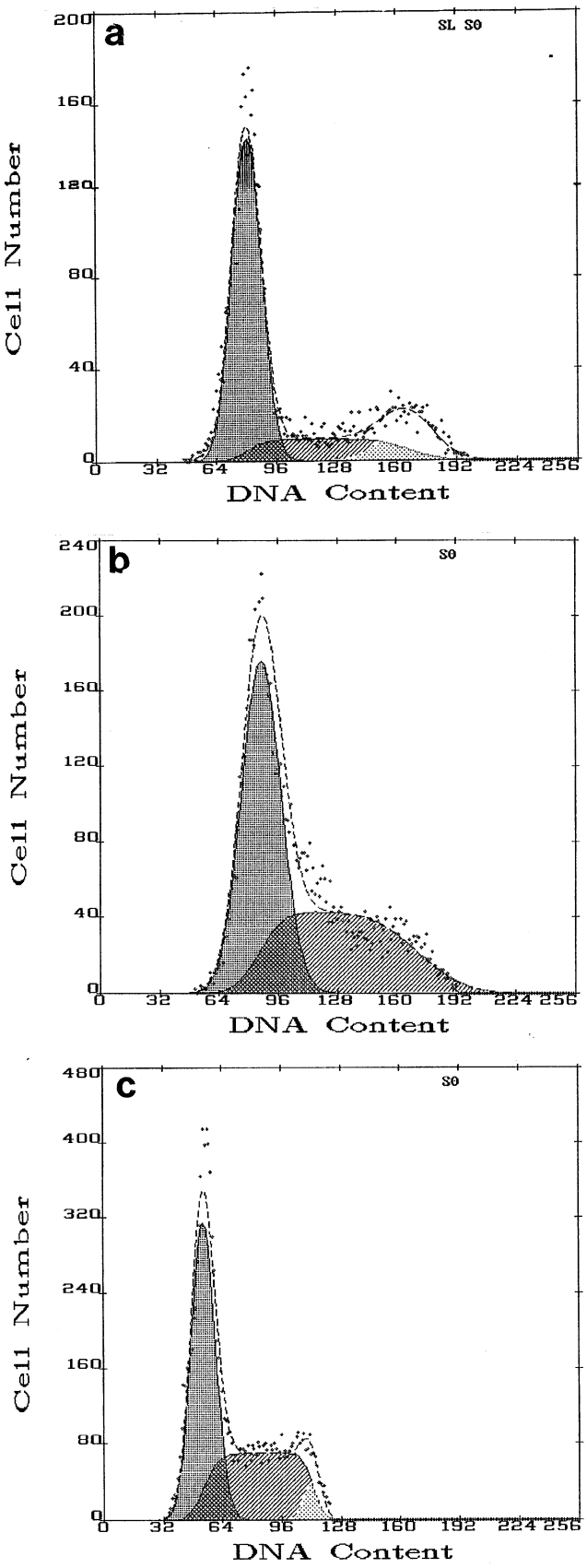

FIG. 3. Cell-cycle analysis by flow cytometry in control cultures (a) and in cultures maintained for 48 hr in the presence of 50 μM DFO (b) or CP20 (c).

TABLE 1. Cell-cycle changes in HepG2 cell-line cultures after 48 hr of exposure to DFO or CP20

	Cell cycle distribution (%)		
	G0-G1	S	G2-M
Duration of exposure (hr)		48	
Iron chelator			
0 μM	65.1 ± 1.9	19.4 ± 2.5	15.5 ± 4.0
DFO 50 μM	52.7 ± 2.9 ($P < 0.01$)	43.4 ± 4.2 ($P < 0.01$)	2.3 ± 1.5 ($P < 0.01$)
DFO 100 μM	54.2 ± 10.1 (NS)	40.3 ± 6.5 ($P < 0.01$)	5.6 ± 6.3 ($P < 0.05$)
CP20 50 μM	50.7 ± 13.3 (NS)	39 ± 4.6 ($P < 0.01$)	13.9 ± 3.6 (NS)
CP20 100 μM	43.8 ± 5.2 ($P < 0.01$)	38 ± 4.4 ($P < 0.01$)	18 ± 4.6 (NS)

Values are means ± SEM; NS: not significant.

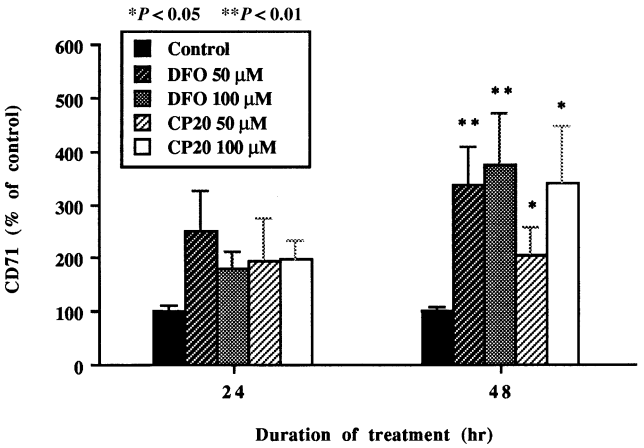

FIG. 5. CD71 positive cell counting in HepG2 control cultures or cultures maintained for 24 and 48 hr in the presence of DFO or CP20 (50 or 100 μM).

Iron Metabolism Study

In order to analyze iron cellular metabolism, we measured iron uptake by the cells, expression of the transferrin receptor, and ferritin intracellular concentration. Our results showed that 50 or 100 μM CP20 or DFO prevented iron uptake by the cells; the effect of the chelators was highly significant for the two concentrations used ($P < 0.001$) and no dose-dependency was observed (Fig. 4). Analysis of the transferrin receptor expression performed by flow cytometry showed no significant increase in its expression at 24 hr of treatment with the chelators; however, a significant increase was reported after 48 hr of exposure to the chelators (DFO 50 or 100 μM, $P < 0.01$; CP20 50 or 100 μM, $P < 0.05$; Fig. 5). This effect was confirmed by Northern blot analysis of the transferrin receptor mRNA, which increased after 24 or 48 hr of exposure to the two concentrations of DFO or CP20 (Fig. 6). At 24 hr, the transferrin mRNA level was increased 2.5- or 3.2-fold over the control value (Fig. 6) in the presence of 50 or 100 μM DFO or CP20, respectively. In addition, we observed a significant, but not dose-dependent, decrease in intracellular ferritin after 24 and 48 hr of treatment with 50 or 100 μM DFO or CP20 (at 48 hr: DFO 50 or 100 μM, $P < 0.001$; CP20 50 or 100 μM, $P < 0.001$; Fig. 7).

Toxicity Analysis

We analyzed LDH release and MTT reduction in cultures maintained for 24 or 48 hr in the presence of 50 or 100 μM DFO or CP20. At 24 hr of exposure, the chelators were without effect on LDH release (Fig. 8) and MTT reduction (data not shown). At 48 hr of treatment, we observed a significant increase in the extracellular LDH (DFO 50 or 100 μM, $P < 0.05$; CP20 50 or 100 μM, $P < 0.05$; Fig. 8) and a significant decrease in MTT reduction (data not shown) as compared to control cultures.

DISCUSSION

In genetic hemochromatosis, it is very likely that iron plays an important role in the development of hepatic lesions and hepatocellular carcinoma [5, 8, 13]. In primary rat hepatocyte cultures stimulated by epidermal growth factor/pyruvate, we previously demonstrated that iron was able to induce DNA synthesis [24]. Several studies have suggested that limiting iron uptake by tumor cells may be one strategy to prevent cellular proliferation [12]. Drugs that can exert profound antiproliferative effects are used in many clinical situations. Often, these agents inhibit cell growth at par-

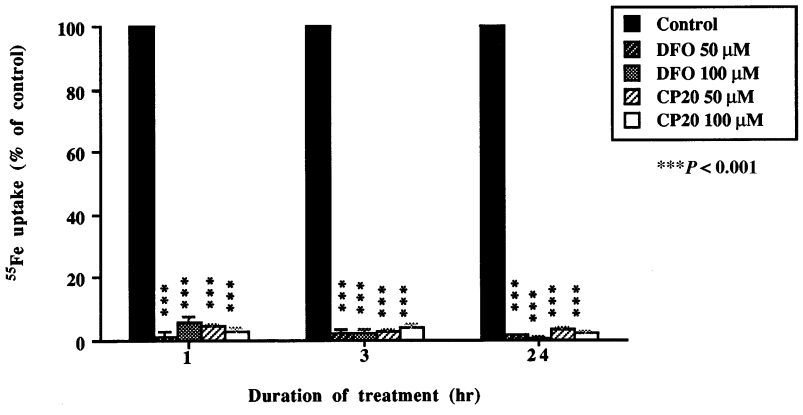

FIG. 4. ^{55}Fe uptake in HepG2 control cultures or cultures maintained for 1, 3 and 24 hr in the presence of DFO or CP20 (50 or 100 μM).

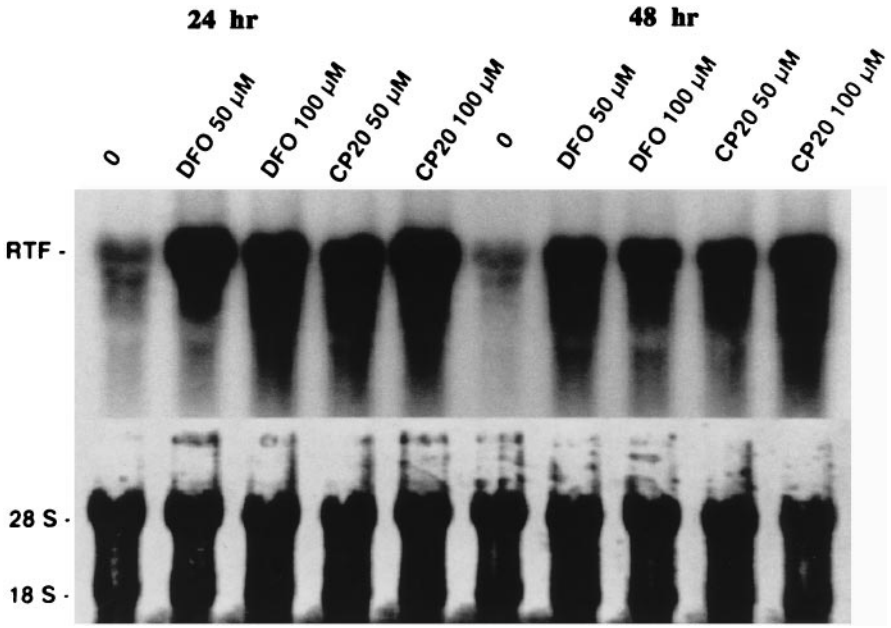

FIG. 6. Northern Blot analysis of human transferrin receptor in HepG2 control cultures (0) or cultures maintained for 24 and 48 hr in the presence of DFO or CP20 (50 or 100 μM).

ticular points in the cell cycle, and attempts have been made to define the molecular basis for such an inhibitory effect. However, it is well known that chelation therapy with DFO has the disadvantage of being inactive when administered orally, due to its poor intestinal absorption. Consequently, in order to cause sufficient iron excretion, DFO has to be given through daily and prolonged s.c. infusion.

In the present study, we have investigated the effect of an oral iron chelator, deferiprone, recently proposed for the treatment of iron overload in thalassemic patients [18, 19]. We have analyzed the effect of CP20 and DFO on HepG2 cell line proliferation, maintained in the absence of FCS, by measuring methyl-thymidine incorporation into DNA and analyzing cell cycle by flow cytometry. We observed, after 48 hr of incubation, a significant decrease in methyl-thymidine incorporation in the presence of both CP20 and DFO when compared to the control cultures. The addition of 50 and 100 μM CP20 or DFO blocked the cell-cycle progression in S phase as attested by the increased percentage of cells in this phase. In addition, a decreased percentage of cells in G0-G1 phase was observed in the presence of the two chelators, while only DFO decreased the percentage of cells in G2-M phase. Taken together, these results suggest that iron chelators may inhibit HepG2 cell proliferation by modifying intracellular iron metabolism. One plausible link between iron and cell proliferation is the ribonucleotide reductase enzyme, which produces the four deoxyribonucleotides from the corresponding ribonucleo-

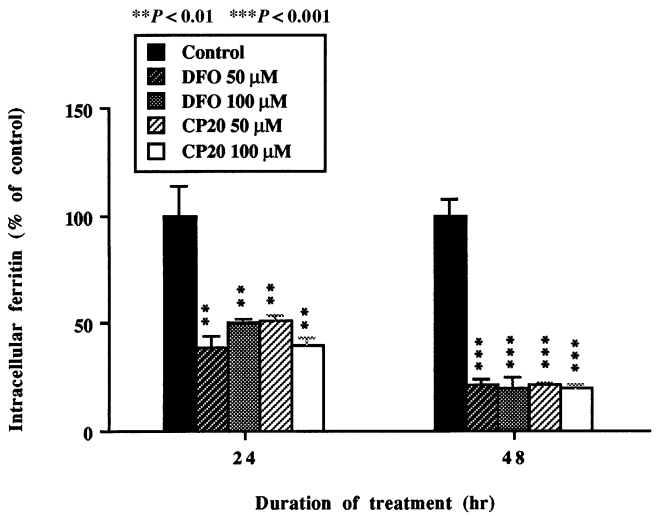

FIG. 7. Intracellular concentrations of ferritin in HepG2 control cultures or cultures maintained for 24 and 48 hr in the presence of DFO or CP20 (50 or 100 μM).

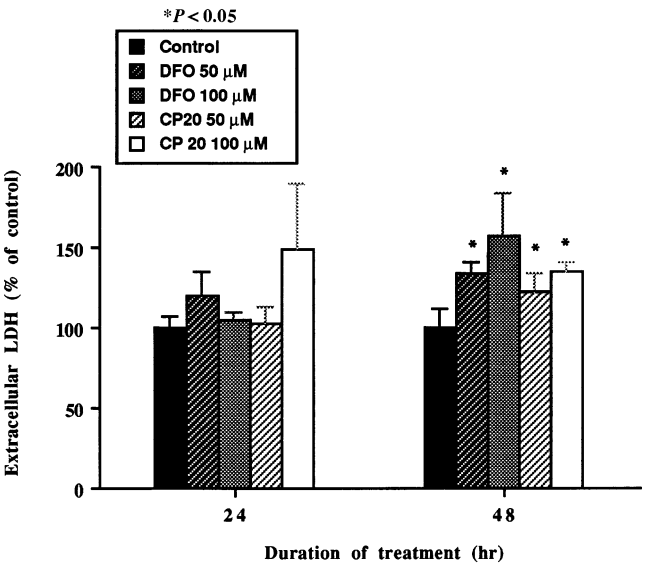

FIG. 8. Extracellular LDH activity in HepG2 control cultures or cultures maintained for 24 and 48 hr in the presence of DFO or CP20 (50 or 100 μM).

tides. Moreover, enzyme activity is iron-dependent and correlates with the cell growth rate [25]. Inhibitors of ribonucleotide reductase blocked DNA synthesis and cell replication. One such inhibitor is DFO, which is thought to act by withholding iron from the M2 subunit of the enzyme [26]. Hydroxyurea is also known to be a ribonucleotide reductase inhibitor, because it blocks cells in early S phase of the cell cycle [27]. Therefore, we have exposed cells to hydroxyurea for 24 hr. The analysis of the effect of CP20 and DFO on DNA synthesis in the presence of hydroxyurea was investigated by measuring methyl-thymidine incorporation into DNA. CP20 and DFO were found to enhance the inhibitory effects of hydroxyurea, as compared to hydroxyurea-treated control cultures. Our results suggest that iron chelators (CP20 and DFO) alone did not completely inhibit the ribonucleotide reductase enzyme since a synergistic action between hydroxyurea and the chelators was noticed. The possibility that iron chelators exert a different blockage from one cell type to another has already been reported in neuroblastoma and leukemia cell lines [15, 28]. However, the present data did not allow us to compare the CP20 and DFO inhibitory effect on cell proliferation, because the two chelators were not added to the culture medium at comparable stoichiometry concentrations.

In order to determine the effects of the two iron chelators on iron metabolism in HepG2 cell line, we have analyzed their impact on iron uptake, transferrin receptor expression and intracellular ferritin concentration. CP20 was effective, as was DFO, in preventing iron uptake by the cells. The high activity of CP20 in preventing the proliferation of tumor cells suggested that this chelator may be useful in the treatment of several neoplastic cells such as DFO [10, 15, 18, 30–32]. The decreased level of the intracellular "regulatory iron pool" by chelation induces transferrin receptor expression as attested by the increase of binding sites at the cell surface and the mRNA level of transferrin receptor in the present study. Because transferrin receptors are maximally induced in the late G1 phase and remain induced during the S phase, an additional factor for transferrin receptor increase following iron deprivation may be cell cycle arrest in S phase [33]. In parallel, CP20 and DFO induced a dramatic decrease in the intracellular ferritin concentration in the presence of 50 and 100 μM DFO or CP20. It is well known that an increase in iron uptake is observed during cell proliferation, since neoplastic cells need a high level of iron. However, cellular iron depletion decreases DNA synthesis, while an increase in transferrin receptor expression [34] and a decrease in ferritin production [35] were reported. In fact, the chelatable iron pool appears to play an important role in the control of cell proliferation and survival by regulating transferrin receptor expression and ferritin production. This overexpression of transferrin receptor could be related to cellular iron depletion, which increases the iron-regulatory protein-iron-regulatory element (IRP-IRE) affinity, increasing transferrin mRNA stability [36].

We have analyzed the toxicity of these iron chelators and their effects on cell viability. We observed that in the presence of DFO or CP20, LDH release increased and succinate dehydrogenase activity (MTT) decreased significantly after 48 hr of exposure. The toxicity of these drugs correlated to their antiproliferative activity observed in vitro will be useful to enhance the effect of other cytotoxic antitumor agents in the treatment of liver cancer. Indeed, it has been reported that the incorporation of iron chelators in chemotherapy protocols provides additional advantages in the treatment of neuroblastoma [16].

In conclusion, we observed an inhibitory effect of CP20 on DNA synthesis with an arrest of HepG2 cell proliferation in the S phase of the cell cycle. The mechanisms involved in this inhibition are likely multiple and require further investigation.

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