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Report

Increased cytotoxicity and stability of Lipiodol–pirarubicin emulsion compared to classical doxorubicin–Lipiodol: potential advantage for chemoembolization of unresectable hepatocellular carcinoma

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There is no well-defined curative treatment for advanced and unresectable hepatocellular carcinoma. The widely used transarterial chemoembolization (TACE) with a doxorubicin–Lipiodol emulsion has not been shown to improve survival in randomized studies. Further, obstruction of the hepatic artery used in the procedure is badly tolerated in patients with cirrhosis. Drugs with a more rapid penetration into the cancer cells are likely to eliminate the need for obstruction of the hepatic artery. We therefore compared the cytotoxicity of another anthracycline pirarubicin with that of the commonly used doxorubicin. In this report, we show that pirarubicin has a greater *in vitro* cytotoxic effect than doxorubicin on the HepG2 and Hu-H7 human hepatoma cell lines. Pirarubicin emulsion with Lipiodol is more stable at 37°C than doxo-rubicin–Lipiodol. Moreover, pirarubicin accumulates at a greater extent in the oil phase, permitting Lipiodol to act as a slow-releasing vector for the anthracycline. Further, amiodarone, a multidrug resistance inhibitor, was shown to decrease the intrinsic resistance of HepG2 and Hu-H7 cells to both anthracyclines, and the presence of polysorbate 80 in the amiodarone preparation increased the stability of the anthracycline–Lipiodol emulsions. We therefore conclude that pirarubicin is a better candidate for TACE than doxorubicin. The rapid and increased cytotoxicity of pira-rubicin on hepatoma cancer cells and the stability of the pirarubicin–Lipiodol amiodarone emulsion could avoid the complete obstruction of the hepatic artery by Gelfoam sponges, and provide a better tolerated method of TACE in patients with latent liver insufficiency. [© 2001 Lippincott Williams & Wilkins.]

Key words: Amiodarone, chemoembolization, doxorubicin, hepatocellular carcinoma, Lipiodol, multidrug resistance, pirarubicin.

Introduction

Hepatocellular carcinoma (HCC) associated with hepatitis B and C or alcohol consumption is one of the most common cancers worldwide. Surgery can cure patients with HCC only at an early stage, and there is no well-defined curative treatment for advanced and unresectable HCC.[1] Even though transarterial chemoembolization (TACE) has an antitumoral activity on unresectable hepatocellular carcinoma,[2,3] it has not been shown to increase the overall survival in randomized trials.[4–7] A classical TACE method consists of injecting an emulsion of doxorubicin (0.5–1 mg/kg in 10–20 ml aqueous solution) in an equal volume of iodized oil (Lipiodol) through a catheter placed into the common hepatic artery.[8] The doxorubicin–Lipiodol emulsion distributes and stays for a time in the rich vascular bed of HCC,[9] whereas it quickly passes through the sinusoids vessels of normal liver parenchyma.[10] At the end of the emulsion injection, small pieces of gelatin sponge (Gelfoam) are injected in order to complete the arterial obstruction and to slow down the blood flow. Gelatin sponge

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dissolves in a few days and the hepatic artery becomes permeable again for further cures. However, the transient obstruction of the hepatic artery is often badly tolerated in these patients with frequent liver cirrhosis.[11] Aggravation of the hepatic insufficiency can lead to death, due to the ischemic damage of the residual liver parenchyma and increase of portal hypertension.[12,13] Therefore, despite its antitumoral activity in 30–50% of the HCC,[14] TACE did not increase the overall survival in randomized trials, due to its induced morbidity and mortality.

Chemoembolization with the Lipiodol–anthracycline emulsion alone, but without Gelfoam, is better tolerated but less efficient than the classical procedure.[12,15] Arterial obstruction is probably required to overcome the slow penetration of doxorubicin in HCC cells. Lipiodol emulsion of anticancer drugs with faster and higher activity, and not requiring arterial interruption, could be more efficient and better tolerated than classical doxorubicin–Lipiodol. In this report, we compared the *in vitro* cytotoxicity of two anthracyclines, pirarubicin and doxorubicin, on two cell lines of human HCC in the presence or absence of amiodarone, a multidrug resistance inhibitor,[16] and the *ex vivo* stability of the emulsion of both drugs with Lipiodol.

Materials and methods

Cancer cells and cytotoxicity assay

The HepG2 hepatocellular cancer cell line was obtained from ATCC (Manassas, VA). The HuH-7 hepatoma cell line was described by Nakabayashi *et al.*[17] Both cell lines were cultivated in Dulbecco's minimum essential medium supplemented with 4.5 g/l glucose, 40 mM glutamine and 10% fetal bovine serum. Cells were detached for experiments with a mixture of 1 g/l trypsin and 0.4 g/l EDTA in Hanks' balanced saline solution.

Cells (5×10^4/well) were seeded in 200 μl culture medium in 96-well tissue culture plates and cultivated for 72 h until confluence. After drug incubation, wells were rinsed twice and incubated for 6 additional days in drug-free culture medium, with renewal of culture medium at day 3. Cytotoxicity was measured by a classical MTT colorimetric assay.[18] In brief, cells were incubated for 1 h at 37°C with 100 μl of a solution containing 2 mg/ml tetrazolium blue (Sigma, St Quentin-Fallavier, France) and 2 g/l glucose in phosphate-buffered saline. Precipitated formazan was eluted by 100 μl DMSO. Plates were read at a 492 nm wavelength on an automatic spectrophotometer. Cell survival was expressed as a percent of control untreated cells. Each point was the mean $\pm$ SD of four wells. Two independent experiments were performed.

Drugs

Pirarubicin (Theprubicine) and doxorubicin (Adriblastine) were obtained as powder under their commercial form from Aventis (Paris, France) and Pharmacia Upjohn (St Quentin en Yvelines, France), respectively. Drugs were dissolved (2 mg/ml) in a 50 g/l glucose solution immediately before use. Human serum (group O) was purchased from Sigma. Amiodarone for i.v. use (Cordarone IV) was obtained from Sanofi Synthelabo (Paris, France). Each milliliter of the commercial preparation contains 50 mg amiodarone–HCl, 20.2 mg of benzyl alcohol, 100 mg of polysorbate 80 and water for injection. Lipiodol (Lipiodol Ultrafluide; Guerbet, Aulnay sous Bois, France) is a sterile iodine addition of the ethyl ester of the fatty acid obtained from poppy seed oil (iodine concentration 38 w/w %).

Preparation of Lipiodol–anthracycline emulsion and measurement of its stability

The emulsion was prepared by mixing an equal volume of Lipiodol and the anthracycline solution (2 mg/ml) through a three-way stopcock from one syringe to another (10 passages). When used, amiodarone was mixed at a final concentration of 3 mg/ml of emulsion. Emulsion was poured in glass vials and stability was evaluated at 37°C for 1 h by measuring the rapidity of the separation between the aqueous, emulsion and oil phases.

Anthracycline assay by high-performance liquid chromatography (HPLC)

One hour after the emulsion preparation, vials were centrifuged at 4000 r.p.m. for 8 min to complete the phase separation. Anthracyclines were assayed in the aqueous and oil phase or in the persisting emulsion phase using a reversed-phase HPLC, following a modification of the method of Munck *et al.*[19] Briefly, 100 μl of each phase was homogenized with 25 μl of alkaline buffer (100 mM Na_2HPO_4/30 mM heptanesulfonic acid, pH 8.5) in 400 μl of DMSO. Daunorubicin (Sigma) was added as the internal standard (25 μl of a 5 mg/ml solution). Aliquots of 30 μl of the supernatant were injected into the HPLC system (Waters, St Quentin, France) with an automatic injector (Autosampler 717; Waters), a 660-MS pump (Waters) and a fluorescence detector (Waters; 470, excitation 251 nm, detection 517 nm). The mobile phase consisted of ammonium formiate buffer (0.16 M, pH 4)

and acetonitrile (68:32, v/v) at a flow rate of 1.5 ml/min. Reverse phase was a Microbondapak column (C18, 10 μm, 3.9 × 300 mm; Waters). Under such conditions, the retention time of doxorubicin, daunorubicin and pirarubicin was 4.7, 7.6 and 10.8 min, respectively.

Results

Cytotoxicity of pirarubicin and doxorubicin on hepatoma cells

As anthracycline–Lipiodol emulsion is rapidly labile, drug cytotoxicity was assayed after a short 5-min incubation. Pirarubicin was more cytotoxic than doxorubicin both on HepG2 and Hu-H7 hepatoma cells (Figure 1A). Amiodarone increased the anthracycline cytotoxicity on both cell lines. Incubation in pure human serum resulted in a slightly reduced cytotoxicity of both anthracyclines—pirarubicin remaining more active than doxorubicin (Figure 1B).

Stability of the anthracycline–Lipiodol emulsion

Lipiodol emulsion carries the anthracyclines up to the microvessels of HCC, slows down the blood flow and increases the contact time of emulsified drugs with tumor cells. Emulsion stability is probably an important

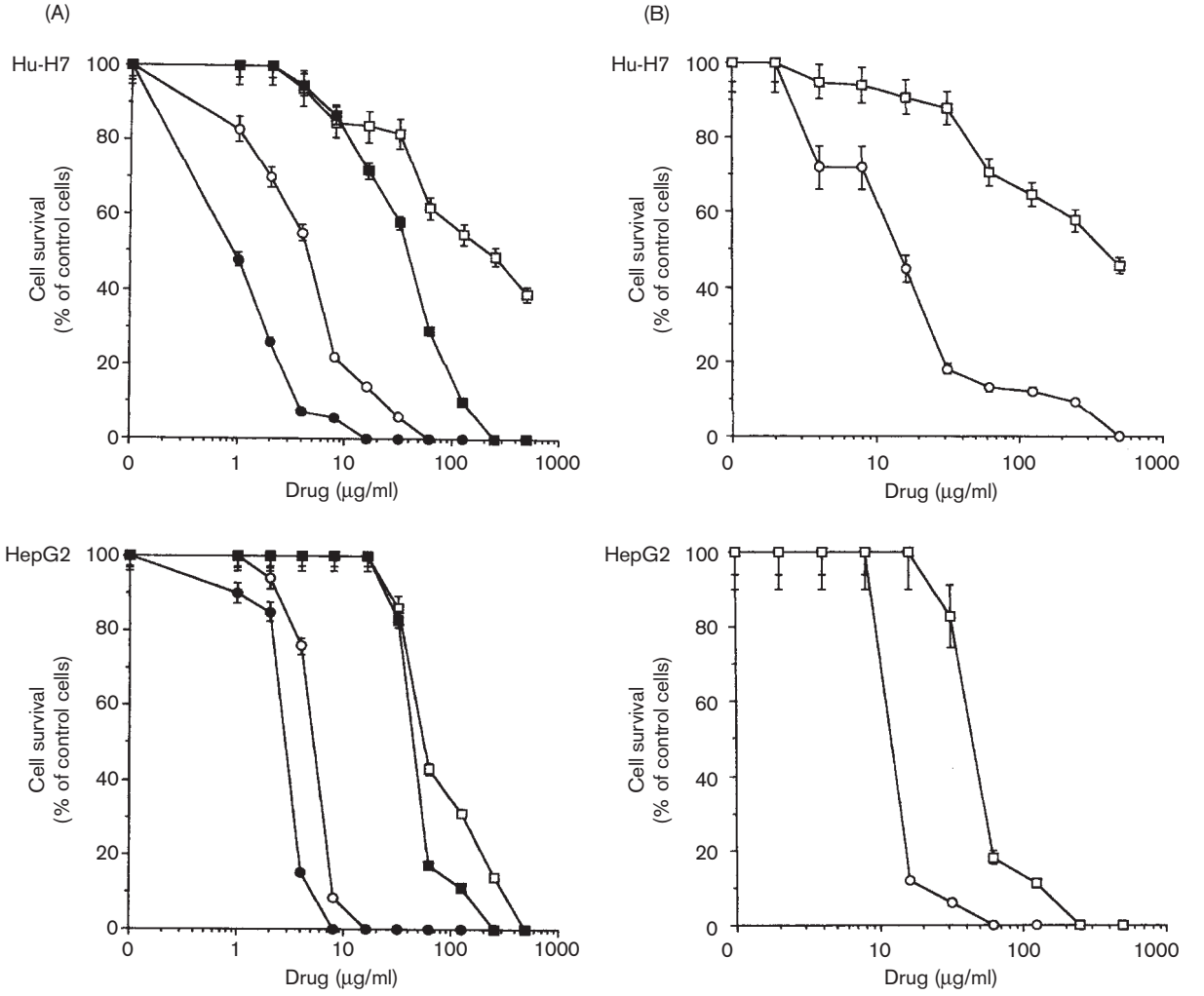

Figure 1. Comparative cytotoxicity of pirarubicin and doxorubicin. Confluent HepG2 and Hu-H7 human hepatocarcinoma cells were briefly incubated for 5 min with pirarubicin (circles) or doxorubicin (squares) in the presence (solid symbols) or not (open symbols) of 5 μg/ml amiodarone. Drugs were diluted in Dulbecco's minimum essential medium (A) or human serum (B). Cells were rinsed and then cultivated again for 6 days in drug-free culture medium. Survival was assayed by a MTT test 6 days after drug exposure. Each point was the mean ± SD of four wells (two independent experiments).

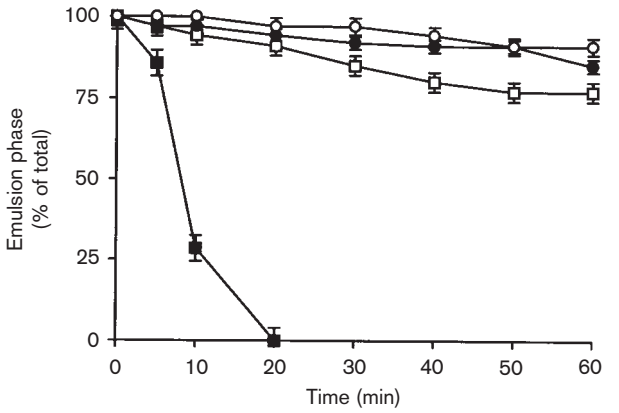

Figure 2. Stability of the Lipiodol–anthracycline emulsions. Pirarubicin (circles) or doxorubicin (squares) was diluted in 50 g/l glucose solution (2 mg/ml) and emulsified with an equal volume of Lipiodol. Amiodarone (3 mg/ml) was added (open symbols) or not (solid symbols) to the anthracycline emulsion. Height of the emulsion phase was reported as a fraction of the total height of the mixture.

parameter for the antitumor effect of chemoembolization. Oil and aqueous solution separated quickly into two phases for the Lipiodol–doxorubicin emulsion (Figure 2). In contrast, phase separation was slower for pirarubicin–Lipiodol with three distinct phases (oil, aqueous solution and persisting emulsion) 1 h after the preparation. Addition of amiodarone for i.v. use increased the emulsion stability both for doxorubicin and pirarubicin. In such conditions, the emulsion of pirarubicin–Lipiodol was stable for more than 4 weeks.

Phase distribution of anthracyclines

Anthracycline concentration was assayed by HPLC 1 h after the emulsion preparation in the different phases (oil, aqueous and persisting emulsion) obtained after centrifugation (Figure 3). Most of drugs remained in the aqueous phase. Pirarubicin concentration was higher ($\times$6-fold) in the oily phase than doxorubicin. No Lipiodol emulsion persisted at 1 h for doxorubicin. In contrast, pirarubicin was still in emulsion with Lipiodol at 1 h at a concentration of 745 mg/l. Addition of amiodarone increased the concentration of both anthracyclines in the oil phase—pirarubicin remaining more liposoluble than doxorubicin. Amiodarone stabilized the Lipiodol emulsion for both drugs.

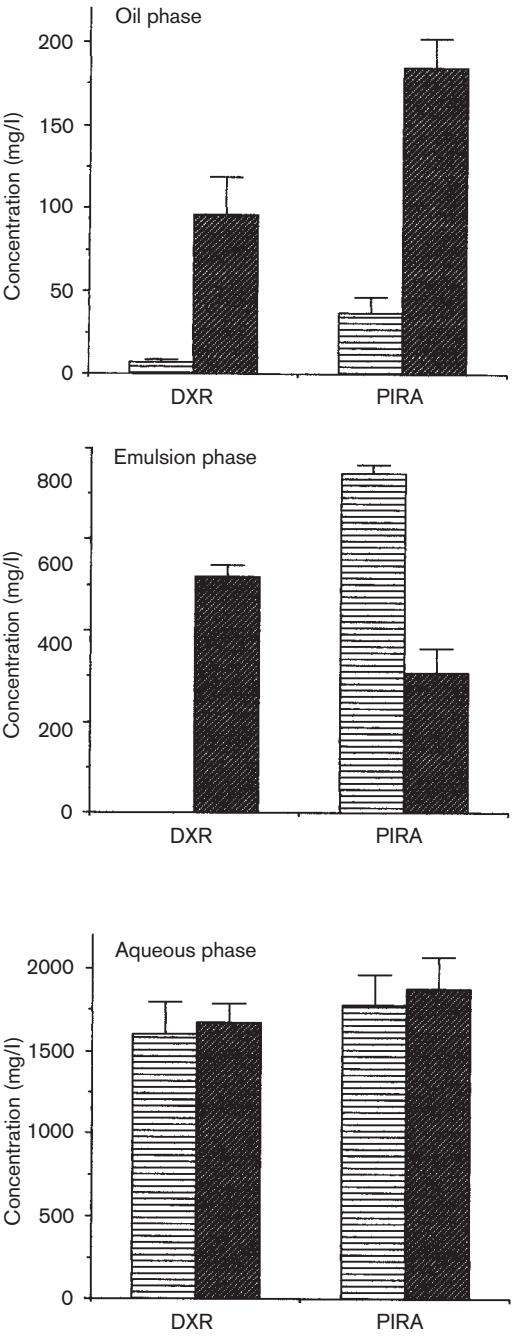

Figure 3. Phase distribution of anthracyclines. Doxorubicin (DXR) or pirarubicin (PIRA) were assayed by HPLC in the aqueous, oily or persisting emulsion phase, 1 h after mixing with Lipiodol, in the presence (▨) or not (▤) of 3 mg/ml amiodarone.

Discussion

Pirarubicin, in contrast to doxorubicin, accumulates rapidly into cancer cells and is more cytotoxic due to its greater lipophilicity.[19–21] In a phase I trial for intraarterial chemotherapy (without Lipiodol) of liver metastases from colorectal cancer, a tumor response was registered in four out of nine evaluable patients.[23,24] The drug was well tolerated with no

hepatic toxicity and a dose-dependent hematologic toxicity for doses above 100 mg/m^2. Izumi *et al.*[24] have replaced classical doxorubicin by pirarubicin (30–60 mg) for chemoembolization of HCC, along with Gelfoam arterial obstruction. A partial response was registered in seven of 10 patients and a minor response in two. Only one patient showed no change. Serum α-fetoprotein levels decreased in nine of the 10 patients. Leukopenia below 2000/mm^3 was not observed in any of the patients. No other serious side effect was observed. From these results, the authors suggested that pirarubicin could be a useful chemotherapeutic agent for HCC. Ueno *et al.*[25] have used pirarubicin in combination with cisplatin for TACE. This regimen was more effective than the association of doxorubicin, mitomycin and cisplatin. The cumulative survival rates for the pirurabicin–cisplatin and the doxorubicin–mitomycin–cisplatin groups were 29.8 and 16.3%, respectively, at 3 years; 16.8 and 4.1%, respectively, at 5 years.

Our present results show that pirarubicin could be a better choice than doxorubicin for TACE of HCC. The *in vitro* cytotoxicity of pirarubicin was higher for the two HCC lines tested. Further, in contrast to the doxorubicin–Lipiodol emulsion which separates rapidly,[26,27] emulsion of pirarubicin with Lipiodol was relatively stable for 1 h. This higher emulsion stability would be probably advantageous for delivering the anticancer agent into the rich tumor microvascular bed of HCC and to increase the contact time of the dispersed aqueous phase with the cancer cells. Moreover, pirarubicin accumulates at a greater extent than doxorubicin in the oily phase. Therefore Lipiodol that persists for weeks or months in hepatoma[28] could be an interesting slow-releasing vector.[29]

In agreement with the recent reports that *mdr1* is intrinsically expressed by hepatoma cells,[30] the cytotoxic effect of both pirarubicin and doxorubicin was significantly increased on both hepatoma cell lines by amiodarone, a potent inhibitor of the P-glycoprotein or *mdr1*. Moreover, amiodarone for i.v. use was shown to increase the stability of both anthracyclines with Lipiodol. This effect is due to polysorbate 80, an emulsifier used for the i.v. formulation of amiodarone. Amiodarone being both an emulsifier for pirarubicin–Lipiodol and an inhibitor of multidrug resistance, its use should be evaluated in patients. Intravenous amiodarone is used for the treatment of cardiac arythmias at a dose of 150–300 mg in short infusions and is generally well tolerated. Therefore arterial injection of 150 mg amiodarone (3 ml) mixed with pirarubicin (50 mg/m^2) and Lipiodol could be used in patients with unresectable HCC.

Conclusion

The rapid and complete cytotoxicity of pirarubicin on HCC and the stability of the pirarubicin–Lipiodol emulsion could avoid the complete obstruction of the hepatic artery by Gelfoam sponges, with probably a better tolerance in these patients with latent liver insufficiency. Phase I studies are planned to evaluate the tolerance of TACE (without Gelfoam) using pirarubicin–Lipiodol alone or mixed with amiodarone on unresectable HCC.

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