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A Cannabinoid Anticancer Quinone, HU-331, Is More Potent and Less Cardiotoxic Than Doxorubicin: A Comparative in Vivo Study

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ABSTRACT

Several quinones have been found to be effective in the treatment of some forms of cancer; however, their cumulative heart toxicity limits their use. The cannabinoid quinone HU-331 [3*S*,4*R*-*p*-benzoquinone-3-hydroxy-2-*p*-mentha-(1,8)-dien-3-yl-5-pentyl] is highly effective against tumor xenografts in nude mice. We report now a comparison of the anticancer activity of HU-331 and its cardiotoxicity with those of doxorubicin in vivo. General toxicity was assayed in Sabra, nude and SCID-NOD mice. The anticancer activity in vivo was assessed by measurement of the tumors with an external caliper in HT-29 and Raji tumor-bearing mice and by weighing the excised tumors. Left ventricular function was evaluated with transthoracic echocardiography. Myelotoxicity was evaluated by blood cell count. Cardiac troponin T (cTnT) plasma levels were determined by immunoassay. HU-331 was found to be much less cardiotoxic than doxorubicin. The control and the HU-331-treated groups gained weight, whereas the doxorubicin-treated group lost weight during the study. In HT-29 colon carcinoma, the tumor weight in the HU-331-treated group was 54% smaller than in the control group and 30% smaller than in the doxorubicin-treated group. In Raji lymphoma, the tumor weight in the HU-331-treated group was 65% smaller than in the control group and 33% smaller than in the doxorubicin-treated group. In contrast to doxorubicin, HU-331 did not generate reactive oxygen species in mice hearts (measured by protein carbonylation levels and malondialdehyde levels). In vivo, HU-331 was more active and less toxic than doxorubicin and thus it has a high potential for development as a new anticancer drug.

Quinones of various chemical families serve as biological modulators (Thomson, 1987; McIntire, 1998; Meganathan, 2001), and both natural and synthetic quinones are widely used as drugs (Lee, 1999; Begleiter, 2000). Anthracyclines, a large group of quinonoid compounds produced by different strains of *Streptomyces*, exert antibiotic and antineoplastic effects, and they are used to treat several forms of cancer (Begleiter, 2000). The best-known members of this family are daunorubicin and doxorubicin, the first identified anthracyclines (Di Marco et al., 1981). Other quinones are also used as anticancer drugs. Mitomycin C and streptonigrin, produced by *Streptomyces*, and the synthetic epirubicin and mitoxantrone are well known examples (Arcamone and Cassinelli, 1998). Although these and other quinonoid compounds are effective in the treatment of many different forms of cancer, their side effects, the most severe being cumulative heart toxicity, limit their use (Thomas et al., 2002; Zucchi et al., 2003; Schimmel et al., 2004). The development of quinonoid compounds that display antineoplastic activity, but that are less toxic, is a major therapeutic goal.

We have reported the synthesis of a new anticancer quinone, HU-331 (Fig. 1), from cannabidiol, one of the most abundant cannabinoids of *Cannabis sativa* (Kogan et al., 2004). HU-331 was found to be highly effective against tumor xenografts in *nude* mice. It is strongly antiangiogenic, both in vitro and in vivo (Kogan et al., 2006), making this compound a promising scaffold for new antiangiogenic drugs. It specifically inhibits topoisomerase II (Kogan et al., 2007). Here, we report that HU-331, although more active than doxorubicin in a HT-29 colon carcinoma model in nude mice and a Raji model in SCID-NOD mice, is significantly less cardiotoxic.

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ABBREVIATIONS: LVEDD, left ventricular end diastolic diameter; LVESD, left ventricular end systolic diameter; IVSWD, interventricular septal wall diameter; LV, left ventricular; cTnT, cardiac troponin T; Abs, absorbance; MDA, malondialdehyde; EF, ejection fraction; FS, fractional shortening; HU-331, 3*S*,4*R*-*p*-benzoquinone-3-hydroxy-2-*p*-mentha-(1,8)-dien-3-yl-5-pentyl.

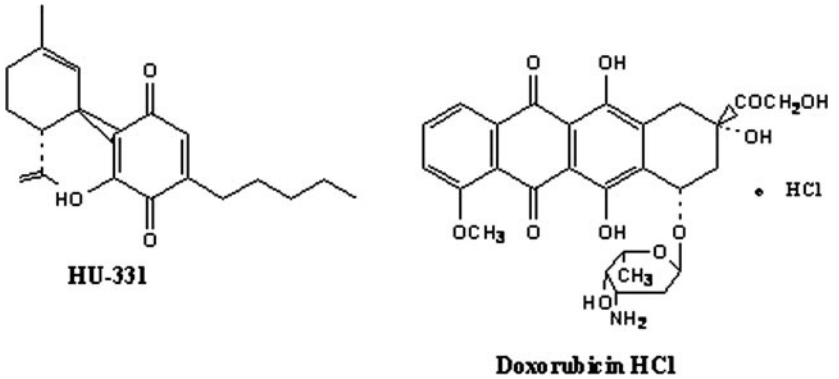

Fig. 1. Structures of HU-331 and doxorubicin.

Materials and Methods

General Toxicity. For determination of toxicity 21 Sabra mice (males, 6–8 weeks old) were divided into three groups of seven mice. The first group received an i.p. injection of vehicle only, the second group received 1.5 mg/kg doxorubicin once a week for 2.5 months, and the third group received 7.5 mg/kg HU-331 once a week for 2.5 months. The mice were weighed every week.

Cardiac Function Assessment. Left ventricular function was evaluated by transthoracic echocardiography at the beginning of the study and before sacrifice. The investigator was blinded both to the treatment and to the control groups. Mice were lightly sedated with an i.p. injection of midazolam. Echocardiography was performed 10 min after initiation of sedation to limit anesthesia-induced impairment of cardiac function (Roth et al., 2002). Echocardiography was performed using a Vivid 7 ultrasound scanner (GE Healthcare, Horten, Norway) with a 13-MHz transducer. Images were stored on magnetic optical disks. Two-dimensional and left ventricle M-mode measurements were taken in two separate 3- to 4-min sessions. The heart was first imaged in the two-dimensional mode in the parasternal short axis view at sweep speed of 150 mm/s. From this mode, an M-mode cursor was positioned perpendicular to the interventricular septum and the left ventricular posterior wall at the level of the papillary muscles. From the M-mode, the left ventricular wall thickness and chamber dimensions were measured.

Image Analysis. All the measurements were performed using the leading-edge method, as recommended by the American Society of Echocardiography (Sahn et al., 1978). For each mouse, three to five values for each measurement were obtained and averaged for evaluation. Two physicians trained in cardiac echocardiography performed the studies. They were blinded to the experimental groups. The left ventricular end diastolic diameter (LVEDD), left ventricular end systolic diameter (LVESD), interventricular septal wall diameter (IVSWD), and left ventricular posterior wall diameter (LVPWD) at end diastole were measured from the M-mode tracing. LV fractional shortening, the percentage of change in LV cavity dimensions, was calculated using the following equation: fractional shortening (%) = [(LVEDD − LVESD)/LVEDD] × 100. Ejection fraction represents stroke volume as a percentage of end diastolic LV volume, and it was calculated from the following equation: ejection fraction (%) = [(LVEDD^3 − LVESD^3)/LVEDD^3] × 100.

Cardiac Troponin T Plasma Levels. Blood was drawn from the mice by heart puncture (approximately 1.5 ml), and then it was transferred to 4-ml plastic tubes with 0.054 ml of 0.015% K_3EDTA. The tubes were centrifuged for 5 min at 3500 rpm. The plasma samples were aliquoted into ice-cooled tubes, and subsequently they were frozen and stored at −70°C until determination. Cardiac troponin T (cTnT) was measured with a third-generation cardio-specific assay (Elecsys Troponin T STAT immunoassay; Roche Diagnostics, Meylan, France) (Baum et al., 1997). The lower limit of detection of this assay was 0.01 ng/ml (range 0.01–25 ng/ml).

Anticancer Activity in Vivo. Procedures involving animals and their care were conducted in conformity with institutional guidelines that are in compliance with international laws and policies and that were approved by the institutional ethics committee. HT-29 colon carcinoma and Raji B-cell lymphoma cancer cell lines were chosen for HU-331 comparison with doxorubicin. For the experiment with HT-29 cells, 30 male nude mice (6–8 weeks old) were used. The HT-29 cancer cells were trypsinized, counted, dispersed in RPMI 1640 medium, without phenol red and without fetal calf serum, and injected subcutaneously into the lower flank (0.5×10^6 cells/0.1 ml of RPMI 1640 medium/mouse).

For the experiment with Raji cells, 30 male SCID-NOD mice (6–8 weeks old) were used. The Raji cancer cells were counted, dispersed in RPMI 1640 medium without phenol red and without fetal calf serum, and they were injected subcutaneously into the lower flank (2.5×10^6 cells/0.1 ml of RPMI 1640 medium/mouse). The mice xenografted with Raji cells received anti-asialo GM1 antibody (Wako Pure Chemicals, Tokyo, Japan) before cell injection, and every 5th day during the first 2 weeks, to neutralize the natural killer cells.

On day 15 after the cancer cell injections (when palpable tumors were already detectable), the mice were randomly divided into three groups of 10. In the HT-29 experiment, the first group received an i.p. injection of vehicle only (1:19/Tween 80:saline), the second group received 0.83 mg/kg doxorubicin three times a week (2.5 mg/kg/week), and the third group received 5 mg/kg HU-331 three times a week (15 mg/kg/week). In the Raji experiment, the first group received an i.p. injection of vehicle only (1:19/Tween 80:saline), the second group received 4.5 mg/kg doxorubicin once a week, and the third group received 15 mg/kg HU-331 once a week (15 mg/kg/week). Tumors were measured with an external caliper, and their area was calculated by multiplying the length of the tumors by their width. The mice were weighed every week. Echocardiography was performed as described above before the sacrifice, and cTnT plasma levels were measured as described above. The mice were sacrificed, and the tumors were excised and weighed.

Blood Cell Count. To evaluate the myelotoxic effect of HU-331 and of doxorubicin, 30 male Sabra mice (6–8 weeks old) were divided into three groups: the first group received a single i.p. injection of vehicle only (1:19/Tween 80:saline), the second group received 4.5 mg/kg doxorubicin, and the third group received 15 mg/kg HU-331. White blood cells and platelets were counted on day 5 after drug administration by means of an automated blood counter (LH750 Analyzer; Beckman Coulter, Fullerton, CA).

Protein Carbonylation Assay. Fifteen male Sabra mice (8–10 weeks old) were divided into three groups, and they were treated with vehicle, 60 mg/kg i.p. HU-331, or 30 mg/kg i.p. doxorubicin (Nowak et al., 1995). The mice were sacrificed after 24 h, and the hearts were excised and homogenized. The protein concentration of each heart homogenate supernatant sample was estimated using the Bradford assay (Bradford, 1976), and the supernatants were diluted to a 2-mg/ml protein concentration. Protein carbonyls were measured by standard methods (Levine et al., 1994; Reznick and Packer, 1994) with slight modifications. Each diluted sample (100 μl) was mixed with 400 μl of 10 mM 2,4-dinitrophenylhydrazine in 2 N HCl,

and then samples were incubated for 1 h at room temperature in the dark, with stirring every 15 min. Proteins were precipitated by adding 500 μl of 20% trichloroacetic acid followed by centrifugation at 14,000 rpm for 10 min at 4°C. The pellet was resuspended in 1 ml of 10% trichloroacetic acid, and then it was centrifuged again at 14,000 rpm for 10 min at 4°C. The pellet was washed three times with 1 ml of ethyl acetate/ethanol (1:1), and it was dissolved in solution of 500 μl of 6 M guanidine with 0.5 M K_3PO_4, pH 2.5. After centrifuging at 14,000 rpm for 10 min, 250 μl of the supernatant was taken, and absorbance was measured at 370 nm. The molar extension coefficient of 2,4-dinitrophenylhydrazine was used to calculate the concentration of carbonyls (ε = 22,000/10^6 nmol/ml) as concentration = absorbance$_{(370)}/\varepsilon$ = Abs$_{(370)}$/0.022 = Abs$_{(370)}$ × 45.45 nmol/ml. A 2-mg/ml protein concentration was used, and the carbonyls concentration was calculated according to concentration = Abs$_{(370)}$ × 45.45 nmol/ml/2 mg/ml = Abs$_{(370)}$ × 22.725 nmol/mg protein (Reznick and Packer, 1994).

Lipid Peroxidation Assay. Twenty-one male Sabra mice (8–10 weeks old) were divided into three groups, and they were treated with vehicle, 60 mg/kg i.p. HU-331, or 30 mg/kg i.p. doxorubicin (Nowak et al., 1995). After 24 h, the mice were sacrificed. Weighted portions of 125 mg of wet hearts were homogenized on ice with 1 ml of 1.15% KCl, and they were mixed with 2 ml of 0.25 N HCl containing 0.375% (w/v) thiobarbituric acid, 15% (w/v) trichloroacetic acid, and 0.015% (w/v) butylated hydroxytoluene. After incubation at 100°C for 20 min, samples were centrifuged (1500*g* for 5 min), and the absorbance of the supernatant was measured at 532 nm against a blank sample containing 1.15% KCl. An extension coefficient of 1.56 × 10^5 M^{-1} cm^{-1} was used to calculate micromoles of malondialdehyde (MDA) per gram of wet organ (Nowak et al., 1995).

Statistical Analyses. Results shown represent mean ± S.E.M. Statistical analysis was performed by analysis of variance with post hoc analysis by the Student-Newman-Keuls test or by the unpaired Student's *t* test.

Results

General Toxicity in Sabra Mice. At the beginning of the study, the weight of the mice in the three groups did not differ, and it was around 33 g/mouse (Fig. 2A). At the end of the study (after 2.5 months of treatment with vehicle, 1.5 mg/kg doxorubicin once a week, or 7.5 mg/kg HU-331 once a week), the control group and HU-331-treated group significantly gained weight (41.9 ± 0.7 g for the control group and 42.6 ± 1.6 g for the HU-331-treated group), compared with the doxorubicin-treated group, which significantly lost weight (30.2 ± 1.3 g).

Echocardiography in Sabra Mice. The ejection fraction was measured at the beginning of the study and before the sacrifice of the animals. Figure 2B shows that at the beginning of the study, the ejection fraction did not differ between the three groups (95%, normal for mice). At the end of the study (after 2.5 months of treatment with vehicle, 1.5 mg/kg doxorubicin once a week, or 7.5 mg/kg HU-331 once a week), the ejection fraction of the control group and of the HU-331-treated group remained unchanged (96 ± 0.6% for the control group and 96 ± 0.6% for the HU-331-treated group). In contrast, in the doxorubicin-treated group, the ejection fraction was significantly lower (83 ± 1.0%) (the full echocardiography data for Sabra mice is presented in Table 1).

Comparison of the Effects of HU-331 and Doxorubicin on HT-29 Colon Carcinoma in Nude Mice. The drug concentrations and administration protocol chosen for this study (0.8 mg/kg doxorubicin three times a week or 5 mg/kg HU-331 three times a week) were near the maximal concentrations of either drug that mice can tolerate following administration for a period of 1.5 to 2.5 months. At the beginning of the study (tumor area around 0.38 cm^2), there was no difference between the groups either in tumor size, cardiac function, or body weight. At the end of the study (after 2.0 months of treatment with vehicle or the drugs), the mean tumor area of the HU-331-treated group was significantly smaller (1.6 ± 0.3 cm^2) than the control mean tumor area (3.1 ± 0.6 cm^2) (Fig. 3A). The mean tumor area in the doxorubicin-treated group, however, was 2.3 ± 0.1 cm^2, thus being 33% larger than the mean tumor area of the HU-331-treated group and 30% smaller than the mean tumor area of the control group. After sacrifice of the mice, the tumors were excised and weighed. The weight of tumors in the HU-331-treated group (1.5 ± 0.3 g) was significantly smaller than

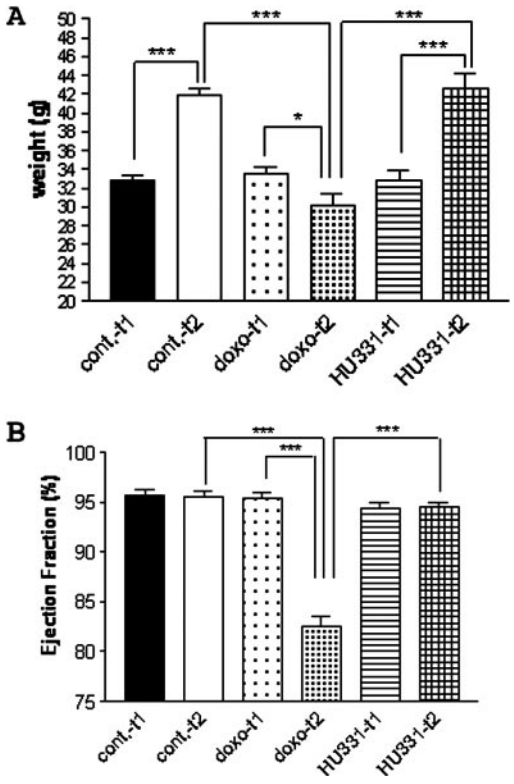

Fig. 2. Toxicity comparison between HU-331 and doxorubicin on Sabra mice. A, weight loss comparison between HU-331 (7.5 mg/kg/week) and doxorubicin (1.5 mg/kg/week). B, cardiotoxicity comparison between HU-331 (7.5 mg/kg/week) and doxorubicin (1.5 mg/kg/week). cont., control; doxo, doxorubicin; t1, time point 1, at the beginning of the study, before mice were treated with any compounds; t2, time point 2, at the end of the study, after mice were treated with the compounds described above for 2.5 months. *, $P < 0.05$; ***, $P < 0.001$.

TABLE 1
Echocardiographic data for Sabra mice treated with vehicle, 7.5 mg/kg/week HU-331, and 1.5 mg/kg/week doxorubicin

	Control	HU-331	Doxorubicin
IVSWD (mm)	0.61 ± 0.04	0.60 ± 0.12	0.66 ± 0.05
LVEDD (mm)	3.5 ± 0.12	3.4 ± 0.16	3.4 ± 0.20
LVESD (mm)	1.2 ± 0.07	1.3 ± 0.09	1.9 ± 0.02**
LVPWD (mm)	0.79 ± 0.03	0.77 ± 0.05	0.78 ± 0.01
EF (%)	96 ± 0.6	95 ± 0.6	83 ± 1.0*
FS (%)	65 ± 4.1	63 ± 1.8	45 ± 3.4*
Heart rate (bpm)	598 ± 22.5	602 ± 12.6	450 ± 38.5

bpm, beats per minute.
* $P < 0.05$; ** $P < 0.01$.

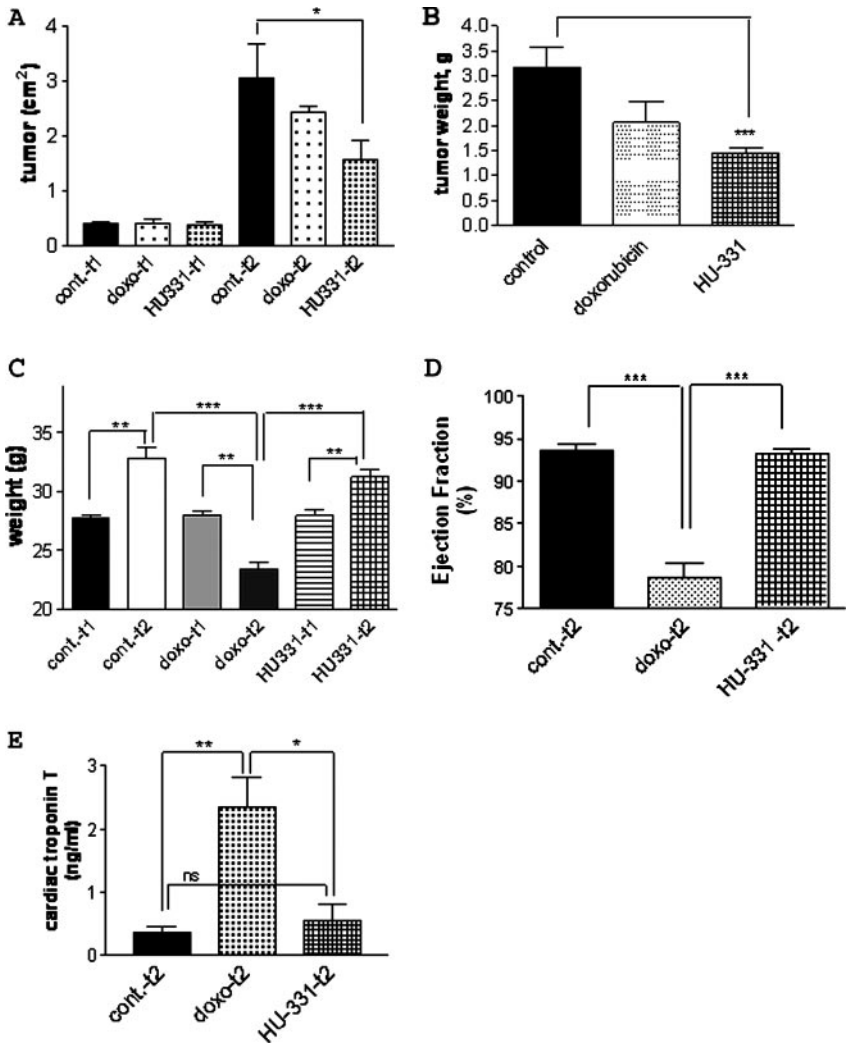

Fig. 3. Toxicity and activity comparison between HU-331 and doxorubicin on nude mice xenotransplanted with HT-29 human colon carcinoma. A, tumor area comparison between the control mice, HU-331-treated mice (15 mg/kg/week), and doxorubicin-treated mice (2.5 mg/kg/week). B, tumor weight comparison at the end of the experiment between control mice, HU-331-treated mice (15 mg/kg/week), and doxorubicin-treated mice (2.5 mg/kg/week). C, weight loss comparison between HU-331 (15 mg/kg/week) and doxorubicin (2.5 mg/kg/week). D, cardiotoxicity comparison between HU-331 (15 mg/kg/week) and doxorubicin (2.5 mg/kg/week) on nude mice, assessed by ejection fraction measurement. E, cardiotoxicity comparison between HU-331 (15 mg/kg/week) and doxorubicin (2.5 mg/kg/week) on *nude* mice, assessed by cardiac troponin T plasma levels. cont., control; doxo, doxorubicin; t1, time point 1, at the beginning of the study, before mice were treated with any compounds (2 weeks after tumor cell injection, when palpable tumors have developed); t2, time point 2, at the end of the study, after mice were treated with the compounds described above for 2 months. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

that in the control group (3.2 ± 0.4 g). The weight of the tumors in the doxorubicin-treated group was 2.1 ± 0.4 g, thus being 30% larger than the mean tumor weight of HU-331-treated group and 35% times smaller than mean tumor weight of the control group (Fig. 3B).

General Toxicity in Nude Mice. The effect of the drugs on the weight of nude mice paralleled that observed in the Sabra mice study described above. At the beginning of the study, the weight did not differ between the three groups, being about 28 g/mouse. At the end of the study, the control group and HU-331-treated group gained weight (control group, 32.9 ± 0.9 g; HU-331-treated group, 31.3 ± 0.6 g), whereas the doxorubicin-treated group lost weight (23.4 ± 0.5 g) (Fig. 3C).

Echocardiography in Nude Mice. At the end of the study (after 2 months) in both the control group and the HU-331-treated group, the ejection fraction remained normal (94 ± 0.7% for the control group and 93 ± 0.6% for the HU-331-treated group), whereas in the doxorubicin-treated group, the ejection fraction was significantly lower (79 ± 1.7%) than in the HU-331 and control groups (Fig. 3D). The full echocardiography data for nude mice are presented in Table 2.

cTnT Plasma Levels in Nude Mice. The concentration of cTnT in the plasma of the doxorubicin-treated group was significantly higher than in the control and HU-331-treated groups (2.4 ± 0.5 ng/ml for doxorubicin-treated group, 0.4 ± 0.1 ng/ml for control group, and 0.6 ± 0.3 ng/ml for HU-331-treated group). The difference between the control group and HU-331-treated group was not statistically significant (Fig. 3E).

TABLE 2
Echocardiographic data for nude mice treated with vehicle, 15 mg/kg/week HU-331, and 2.5 mg/kg/week doxorubicin

	Control	HU-331	Doxorubicin
IVSWD (mm)	0.50 ± 0.01	0.53 ± 0.03	0.57 ± 0.02
LVEDD (mm)	2.92 ± 0.20	2.77 ± 0.21	2.75 ± 0.22
LVESD (mm)	1.2 ± 0.07	1.3 ± 0.09	1.58 ± 0.11*
LVPWD (mm)	0.75 ± 0.05	0.76 ± 0.07	0.65 ± 0.05
EF (%)	94 ± 0.7	93 ± 0.6	79 ± 1.7***
FS (%)	61 ± 0.9	61 ± 1.2	42 ± 2.2***
Heart rate (bpm)	702 ± 32.3	679 ± 45.7	591 ± 10.5*

bpm, beats per minute.
* $P < 0.05$; *** $P < 0.001$.

Activity against Raji Lymphoma in SCID-NOD Mice. The cardiotoxicity and activity against Raji lymphoma of doxorubicin versus HU-331 were compared in SCID-NOD mice. At the beginning of the study there was no difference between the groups either in tumor size, cardiac function, or body weight. The mice were treated with vehicle, 4.5 mg/kg

doxorubicin once a week, or 15 mg/kg HU-331 once a week. After 3 weeks of treatment, 4 of 10 mice in the doxorubicin-treated group died, and the experiment was stopped. The mice were weighed and sacrificed, the tumors were excised and weighed, and blood was drawn for cTnT measurements. The mean tumor weight of the HU-331-treated group (0.3 ± 0.1 g) was more than two times smaller than that of the control group (0.7 ± 0.1 g). The mean tumor weight in the doxorubicin-treated group was 0.4 ± 0.1 g, thus being 33% larger than the mean tumor weight of the HU-331-treated group and 47% smaller than the mean tumor weight of the control group (Fig. 4A).

General Toxicity in SCID-NOD Mice. The effect of HU-331 and doxorubicin on weight paralleled that observed in the Sabra and the nude mice described above. At the beginning of the study, the weight did not differ between the three groups; it was around 26 g/mouse. At the end of the study, the control group and HU-331-treated group gained weight (control group, 28.1 ± 0.4 g; HU-331-treated group, 27.5 ± 0.5 g), whereas the doxorubicin-treated group lost weight (20.9 ± 1.6 g) (Fig. 4B).

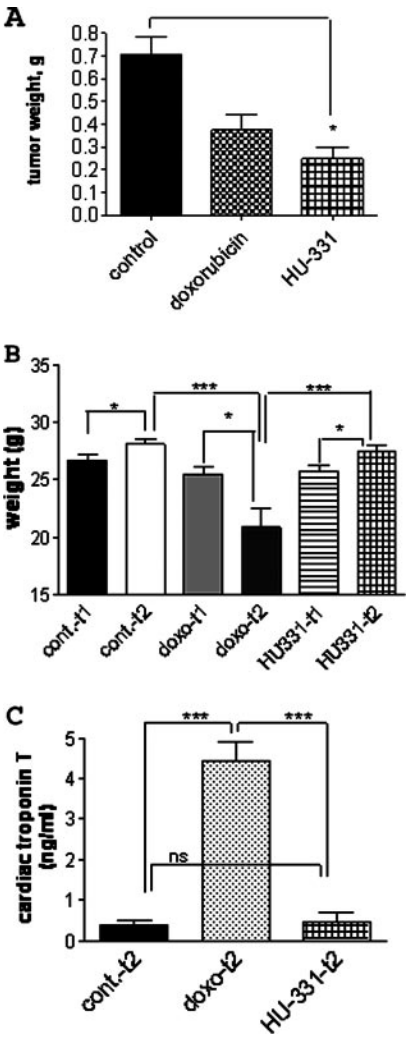

Fig. 4. Toxicity and activity comparison between HU-331 and doxorubicin on SCID-NOD mice xenotransplanted with Raji human B-cell lymphoma. A, tumor weight comparison at the end of the experiment between control mice, HU-331-treated mice (15 mg/kg/week), and doxorubicin-treated mice (4.5 mg/kg/week). B, weight loss comparison between HU-331 (15 mg/kg/week) and doxorubicin (4.5 mg/kg/week). C, cardiotoxicity comparison between HU-331 (15 mg/kg/week) and doxorubicin (4.5 mg/kg/week) measured by cardiac troponin T plasma levels. cont., control; doxo, doxorubicin; t1, time point 1, at the beginning of the study, before mice were treated with any compounds (2 weeks after tumor cell injection, when palpable tumors have developed); t2, time point 2, at the end of the study, after mice were treated with the compounds described above for 2 months. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

cTnT Plasma Levels in SCID-NOD Mice. The concentration of cTnT in the plasma of the doxorubicin-treated group was significantly higher than that of both the control and the HU-331-treated groups (control group, 0.4 ± 0.1 ng/ml; HU-331-treated group, 0.5 ± 0.2 ng/ml; doxorubicin-treated group, 4.4 ± 0.5 ng/ml), whereas the difference between the control group and HU-331-treated group was not statistically significant (Fig. 4C). The echocardiography data showed parallel results (the full echocardiography data for SCID-NOD mice are presented in Table 3).

Blood Cell Count. Blood cell count was performed for mice administered the doses of HU-331 and doxorubicin adopted for the previous study. At a dose of 4.5 mg/kg, doxorubicin significantly suppressed the white blood cells and platelets count, whereas HU-331 had only a small, nonsignificant effect on these parameters even at a dose of 15 mg/kg (Table 4).

Protein Carbonylation Assay. The level of protein carbonyls was measured in hearts of the mice acutely exposed to high doses of HU-331 or doxorubicin. The amount of protein carbonyls in the hearts of mice acutely exposed to 60 mg/kg HU-331 (5.21 ± 0.78 nM) did not differ from that found in control mice (4.73 ± 1.41 nM), whereas in the hearts of mice exposed to 30 mg/kg doxorubicin, it was significantly higher (7.48 ± 0.43 nM) (Fig. 5A).

Lipid Peroxidation Assay. The level of MDA, a byproduct of lipid peroxidation, was measured in hearts of mice acutely exposed to high doses of HU-331 or doxorubicin. The amount of MDA in the hearts of mice acutely exposed to 60 mg/kg HU-331 (1.115 ± 0.045 μmol/g) did not differ from that found in control mice (1.110 ± 0.048 μmol/g), whereas in the hearts of mice exposed to 30 mg/kg doxorubicin, it was significantly higher (1.521 ± 0.056 μmol/g) (Fig. 5B).

Discussion

The data presented here suggest that HU-331 possesses significantly less general toxicity and cardiotoxicity than doxorubicin. Drug concentrations in Sabra mice chosen for this study were nearly the maximal concentrations of both drugs that mice can tolerate following administration for a period of 1.5 to 2.5 months. Although the concentrations of HU-331 were five times larger than those of doxorubicin, HU-331-treated mice gained weight, and their heart function

TABLE 3
Echocardiographic data for SCID-NOD mice treated with vehicle, 15 mg/kg/week HU-331, and 4.5 mg/kg/week doxorubicin

	Control	HU-331	Doxorubicin
IVSWD (mm)	0.625 ± 0.025	0.56 ± 0.174929	0.675 ± 0.047871
LVEDD (mm)	3.05 ± 0.17	3.05 ± 0.12	3.15 ± 0.21
LVESD (mm)	1.3 ± 0.09	1.4 ± 0.08	1.98 ± 0.03***
LVPWD (mm)	0.8 ± 0.04	0.75 ± 0.05	0.78 ± 0.03
EF (%)	91 ± 2.3	91 ± 1.0	75 ± 3.8*
FS (%)	57 ± 4.1	56 ± 1.8	38 ± 3.5*
Heart rate (bpm)	606 ± 28.2	611 ± 8.7	423 ± 49.7*

bpm, beats per minute.
* $P < 0.05$; *** $P < 0.001$.

TABLE 4
Blood cell count in Sabra mice treated with vehicle, 15 mg/kg HU-331 and 4.5 mg/kg doxorubicin

	Control	HU-331	Doxorubicin
WBC ($10^3/\mu l$)	6.64 ± 0.88	5.26 ± 1.21	3.74 ± 0.46*
PLT ($10^3/\mu l$)	1281 ± 40.9	1251 ± 93.9	828.6 ± 140.4*

* $P < 0.05$.

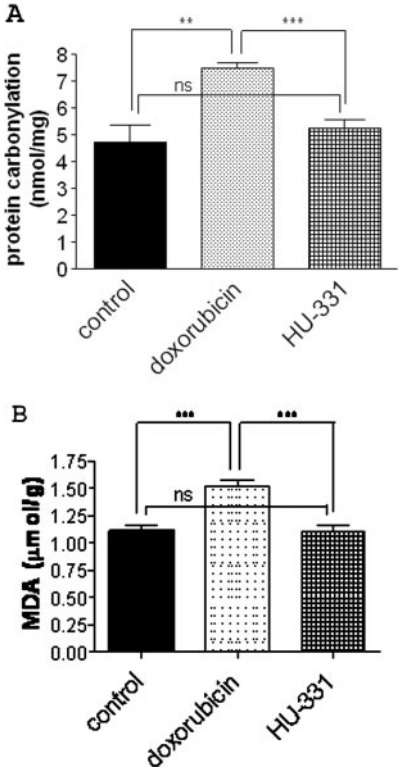

Fig. 5. A, protein carbonylation comparison between acute treatment by 60 mg/kg HU-331 and 30 mg/kg doxorubicin in Sabra mice hearts. B, lipid peroxidation comparison between acute treatment by 60 and 30 mg/kg doxorubicin measured by MDA levels in Sabra mice hearts. **, $P < 0.01$; ***, $P < 0.001$.

was not impaired, contrary to doxorubicin-treated mice, which lost weight and showed heart function impairment. In fact, HU-331-treated mice did not differ from the control mice that were treated with vehicle only.

Similar results were obtained with nude mice treated with HU-331 versus doxorubicin. Because experiments with Sabra mice HU-331 (7.5 mg/kg/week) did not cause any cardiotoxicity, a larger dose of HU-331 was given to nude mice, 15 mg/kg/week, divided into three injections of 5 mg/kg. A dose of doxorubicin (2.5 mg/kg/week) was administered to nude mice, divided into three injections of 0.83 mg/kg. HU-331 exerted a stronger anticancer effect on HT-29 human colon carcinoma xenografts than doxorubicin. The nude mice were weighed every week, and the general toxicity results paralleled those obtained in Sabra mice, namely, the control and HU-331-treated groups gained weight, whereas the doxorubicin-treated group lost weight. The difference in weight (Fig. 3C) between tumor-bearing controls and HU-331-treated groups is probably due to the much larger weight of tumors in the control group.

Echocardiography was performed only at the end of the study, because we were not allowed to take nude mice out of the pathogen-free zone in the animal house to perform echocardiography at the beginning of the study and then return them to the same zone later. Echocardiography showed no impairment in cardiac function following treatment with HU-331 (based on comparable data noted with the control group) versus significant cardiac function impairment elicited by doxorubicin.

The results of echocardiography were strengthened by an additional assay, measurement of the plasma levels of cTnT. Troponins are proteins found in cardiac and skeletal muscle, and the troponin complex (subunits I, T, and C) on the thin filament regulates the force and velocity of muscle contractions. Plasma levels of cTnT are increasingly recognized as potential biochemical markers of subclinical myocardial injury (Lefrak et al., 1973; Henderson and Frei, 1979) useful for detection of anthracycline cardiotoxicity (Herman et al., 2001; Auner et al., 2003). Measurement of cTnT plasma levels has been found to be a valuable tool also in experiments with laboratory animals (O'Brien et al., 1997; Feleszko et al., 2000). In the troponin study, the results obtained with nude mice treated with HU-331 versus doxorubicin paralleled the results obtained in echocardiography.

HU-331 (15 mg/kg once a week) and doxorubicin (4.5 mg/kg once a week) were administered to SCID-NOD mice. The mice were weighed every week, and the general toxicity results paralleled those obtained in Sabra and nude mice—the control and HU-331-treated groups gained weight, whereas the doxorubicin-treated group lost weight. The cTnT plasma levels in this study paralleled the levels measured in *nude* mice, and although the HU-331 and the control groups showed no sign of cardiotoxicity, doxorubicin was highly cardiotoxic. Four of 10 mice in the doxorubicin-treated group actually died because of its toxicity; yet, the tumors in this group were not smaller than those in HU-331-treated group, which showed no signs of weight loss or cardiotoxicity (Fig. 4).

Myelotoxicity is a very serious side effect and is the dose-limiting factor of chemotherapy with doxorubicin. Indeed, at a dose of 4.5 mg/kg, doxorubicin significantly suppressed the white blood cell and platelet counts. In contrast, HU-331 did not have a significant effect on these parameters even at a dose of 15 mg/kg.

As can be seen from these studies, HU-331 is much less cardiotoxic (and also less myelotoxic) than doxorubicin. HU-331 shows anticancer activity at concentrations that cause minimal toxicity, as seen from mouse weight gain, echocardiography, cardiac troponin T plasma levels, and blood cell count. In contrast, doxorubicin is toxic (as indicated by heart function impairment, low blood cell count, and weight loss of the tumor-bearing hosts), even at therapeutic doses, which in our models show activity lower than that of HU-331.

As a quinone, HU-331 should be expected to generate the same reactive oxygen species that have been implicated to explain the cardiotoxicity induced by doxorubicin. Thus, it seemed logical to assay HU-331 for its ability to generate free radicals in the hearts of mice by measuring biochemical surrogates of oxidative stress, such as protein carbonyls. Although HU-331 was previously shown by us to be incapable of generating free radicals in cancer cells (Kogan et al., 2007), we investigated heart tissue, assuming that it could possibly react differently. We checked the level of protein carbonyls, a biochemical surrogate of oxidative stress, in the

hearts of mice acutely exposed to high doses of either HU-331 or doxorubicin. The amount of protein carbonyls in the hearts of mice exposed to 60 mg/kg HU-331 did not differ from that of control mice, whereas in the hearts of mice exposed to 30 mg/kg doxorubicin, it was significantly higher. To strengthen this finding, lipid peroxidation was evaluated in the mice hearts by measuring its by-product MDA. The amount of MDA in the hearts of mice exposed to 60 mg/kg HU-331 did not differ from that of control mice, whereas in the hearts of mice exposed to 30 mg/kg doxorubicin, it was significantly higher. The absence of free radicals formed by HU-331 may be one of the reasons for its lack of cardiotoxicity.

In a previous study, the sensitivity of a BE colon carcinoma cell line and of the HT-29 cell line to HU-331 were compared. The BE colon carcinoma cell line has a point mutation and lacks DT-diaphorase [NAD(P)H:quinone oxidoreductase]; the HT-29 cell line is the same line but without the mutation. The two cell lines were affected by HU-331 to the same extent, which indicates that HU-331 does not act through one-electron reduction (Kogan et al., 2007). Apparently, the one-electron reduction of the quinone moiety in HU-331 is less effective than in doxorubicin, and this effect may be a further reason for the lack of cardiotoxicity of HU-331.

A significant reduction in the heart rate was noted in the doxorubicin-treated groups. This effect has previously been seen, both in patients treated with doxorubicin and in laboratory animals models (Liu et al., 2002; Paiva et al., 2005; Li et al., 2006). It could represent a limitation of the interpretation of the physiological data as the measurement of LV function parameters before and during treatment can be influenced by numerous factors, such as abnormal heart rate, abnormal loading conditions, the presence of cytokine-mediated myocardial depressants, anemic syndrome, infiltration of the myocardium with blasts, sympathetic overdrive, and hyperkinetic circulation. Hence, some authors recommend the use of cardiac troponin T measurement instead of echocardiography (Lipshultz et al., 2004). In our study, both echocardiography and cardiac troponin T measurements were used, and both methods produced parallel results. The two cancer cell lines chosen for treatment with HU-331, HT-29 colon carcinoma and Raji lymphoma, differ in their nature and sensitivity to anthracyclines.

Colorectal cancer is the second most common cause of cancer-related mortality in Western countries, with about 1 million new cases every year diagnosed worldwide and 500,000 patients dying from the disease (Parkin et al., 2005). Resistance of colorectal cancer to established treatment regimens remains one of the major concerns in oncology. Colon carcinomas seem to be very resistant to doxorubicin treatment (Giovanella et al., 1989; Nielsen et al., 1996; Ravizza et al., 2004). The cytotoxic efficacies of doxorubicin on the HT-29 cell line, evaluated by a survival assay, and the nuclear drug concentrations, measured by microspectrofluorometry, were shown to progressively decrease with the augmentation of confluence. Confluence-dependent resistance could explain the high resistance to anthracyclines of some solid tumors, such as colon tumors, in which cancer cells are tightly aggregated (Pelletier et al., 1990). Human colon cancer HT29 cells are very susceptible to multidrug-resistance development (Goldstein, 1996).

Taken together, leukemia, lymphoma, and myeloma constitute the fourth most common form of cancer. For all forms of leukemia, the 5-year survival rate is only 46%. Although Hodgkin's lymphoma is the best-known form of lymphoma, the incidence of Hodgkin's lymphoma is lower than that of non-Hodgkin's lymphoma (American Cancer Society: Cancer Facts and Figures, American Cancer Society, Atlanta, GA, http: // www.cancer.org/docroot/STT/stt_0_2006.asp?sitearea=STT&level=1). Anthracycline-containing chemotherapy regimens including cyclophosphamide, doxorubicin, vincristine, prednisone are now generally considered to be "standard" first line regimen therapy for lymphomas (Dana et al., 1993; Fisher et al., 1993). Despite the difference between these two types of cancer, HU-331 was more active than doxorubicin in treating both types, and it was less toxic for the tumor bearing hosts.

The administration of a combination of anticancer drugs and antiangiogenic agents is known to have a synergistic effect on the in vivo growth of cancer (Yigitbasi et al., 2004). Because HU-331 exerts both an antiangiogenic effect (Kogan et al., 2006) and an anticancer effect (Kogan et al., 2004), HU-331 may prove to be a more versatile drug than doxorubicin. Because in vivo HU-331 is more active on cancer growth and less toxic for the tumor-bearing host than doxorubicin, we think that it has a high potential to develop into a new anticancer drug.

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