Increased Efficacy and Reduced Cardiotoxicity of Metronomic Treatment with Cyclophosphamide in Rat Breast Cancer

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Abstract. *Background/Aim: It has been reported that continuous low-dose (metronomic) administration of cytotoxic drugs may be better tolerated and may have greater antitumor effects than a single high-dose chemotherapy. The aim of this study was to examine the efficacy and cardiotoxicity of metronomic administration of two of the most commonly used anticancer agents, cyclophosphamide (CPA) and doxorubicin (DOX), on an experimental breast cancer of rats. Materials and Methods: Breast tumors were induced in Fisher 344 female rats by implanting Mat B III cells. Rats with tumors were randomized into three groups and were treated with a total dose of 160 mg/kg CPA and a total dose of 12 mg/kg DOX, administered twice per week for four weeks. Control rats were injected with saline according to the same schedule. Echocardiography was performed before the start of treatment and before sacrifice, which took place two weeks after the last injection, when plasma troponin was also measured. Results: The metronomic CPA eradicated the tumors and preserved body weight and echocardiographic parameters. The metronomic DOX slowed tumor growth, but was not able to prevent DOX-induced cardiotoxicity. Conclusion: These results suggest that the success of a metronomic chemotherapy in terms of both efficacy and toxicity depends on the target, the class and the route of administration of the anticancer agent.*

Cardiovascular toxicity is a potential complication of anticancer therapy (1, 2). Cardiac toxicity associated with cancer therapies can range from asymptomatic subclinical abnormalities, including electrocardiographic changes and temporary left ventricular ejection fraction (LVEF) decline, to life-threatening events such as congestive heart failure or acute coronary syndromes (3). Cyclophosphamide (CPA) and doxorubicin (DOX) are highly efficient and widely used anticancer agents. The chemotherapy regimens containing CPA (4) and DOX (5) in the usual treatment protocols using the maximum tolerated dose are often associated with cardiovascular complications.

Cardiac toxicity, including acute lethal myocarditis resulting from high-dose CPA chemoradiotherapy, has been described in numerous reports (6). For CPA-containing regimens with doses ≥150 mg/kg, left ventricle dysfunction has been reported in 7% to 28% of patients (7). CPA-induced cardiomyopathy occurs within the initial two to three weeks after treatment (8). CPA is an inactive prodrug that undergoes a complex metabolic process (9). It is hydroxylated by the hepatic microsomal cytochrome P450 system to its active metabolite, 4-hydroxycyclophosphamide, and its tautomer, aldophosphamide. These intermediates undergo either conversion into acrolein and phosphoramide mustard, which are believed to be the toxic and active metabolites, respectively, or oxidation to the inactive compound carboxyphosphamide. CPA metabolites can react with carboxyl, mercapto, amino, phosphate and hydroxyl groups and can form cross-links with DNA and proteins (10, 11). Interstrand DNA cross-linking has been considered to be the primary action mechanism of CPA and its hydroperoxide derivative, 4-hydroperoxycyclophosphamide (17). CPA is believed to exert its toxic effects through damage to the endocardial capillary endothelium, resulting in increased permeability and microthromboses, which produce extravasation of plasma and red blood cells into the myocardium (12).

Studies evaluating the cumulative probability of DOX-induced heart failure have found rates in the range of 3-5% at doses of 400 mg/m^2 and 7 to 26% at 550 mg/m^2 (13). DOX-induced cardiomyopathy is reported to develop between 0 and 231 days following the final dose of DOX (14). However, delayed development of cardiotoxicity of up to 20

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years following therapy has been reported (15, 16), suggesting that cardiac myocytes are damaged during therapy (17). A number of different mechanisms for DOX action have been proposed, including free radical formation with glutathione depletion and DNA cross-linking (18). In addition to nucleic acids and cellular membranes, the cytotoxic action by anthracyclines involves the cytoskeleton of both tumor cells and cardiomyocytes, causing reduction in the density of myofibrillar bundles (19), disarray and depolymerization of actin filaments (20, 21) and decrease of alpha-actin mRNA abundance (36). DOX-induced apoptosis has been established in several cell lines (22), as well as in the heart, kidney and the intestine of DOX-treated rats (23).

Several studies have suggested that continuous low-dose (metronomic) administration of cytotoxic drugs may have better therapeutic results in addition to reduced toxicity (24, 25).

The aim of this study was to examine the cardiotoxicity and efficacy of metronomic CPA and DOX treatment on experimental breast cancer of rats.

Materials and Methods

Animals, cell culture and treatment. A total of 27 female Fisher 344 rats (NCI, Frederick, MD, USA) weighing 180-200 g were used. All studies were approved by the Animal Care and Use Committee at the Central Arkansas Veterans Healthcare System. The rats were maintained in standard cages (two animals per cage) in the Animal Care facility of Central Arkansas Veterans Healthcare System (Little Rock, AR, USA) and were subjected to a 12-hour dark/light cycle. The rats were randomized into the following four groups and treated as follows: (i) tumor-bearing rats (n=8) treated with a total dose of 150 mg/kg CPA (similar to a dose of 800 mg/m^2) administered intraperitoneally (*i.p.*); eight injections (20 mg/kg each), twice a week for one month, (ii) tumor-bearing rats (n=8) treated with a total of 12 mg/kg DOX (similar to a dose of 65 mg/m^2 for humans) (26), administered eight injections (1.5 mg/kg each); (iii) control tumor-bearing rats (n=6) injected twice per week with saline; and (iv) naïve controls (n=5).

Rat mammary adenocarcinoma 13762 Mat B III cell line was obtained from ATCC (Manassas, VA. USA). The cells were maintained in McCoy's 5A modified medium supplemented with 10% fetal bovine serum (FBS), 25 mM 4-(2-hydroxyethy)-1-piperazineethanesulfonic acid, 26 mM sodium bicarbonate, 100 units/ml of penicillin-streptomycin sulfate and 0.2% gentamycin at 37˚C and 5% CO_2. The Mat B III breast cancer model is a widely used *in vivo* model for studies of breast cancer. The Mat B III cell line is hormonally responsive and metastatic and derives from dimethylbenzanthrene (DMBA)-induced ascites tumor from the Fisher rat (27). Mat B III-induced implantable tumors are highly vascularized, fast growing and highly aggressive mammary tumors (28). This model has the advantage that the cell line is native to the Fisher rat.

Breast cancer was induced in the rats by injecting 1×10^6 cells in 0.2 ml saline into the mammary fat pad of rats anesthetized with 2% isoflurane/oxygen.

CPA monohydrate and DOX hydrochloride (Sigma Chemical Co., St. Louis, MO, USA) diluted in saline were administered *i.p.* into the rats after the tumors reached a size of 0.3-0.5 cm. The median lethal dose (LD_{50}) for CPA (450 mg/kg) (29) and DOX (20 mg/kg) was determined on Fisher 344 rats previously (30). CPA and DOX doses were chosen based on the published clinical data for CPA (600-900 mg/m^2) and DOX (60 mg/m^2) administered every 21 days for 4 cycles (31, 32) and the published data from experimental studies with rats (33, 34).

Rats were monitored daily for tumor growth, body weight, food intake and signs of toxicity. All surviving rats were sacrificed two weeks after the last injection. At sacrifice, tumor volumes and weights were recorded. Tumor volumes were calculated according to the formula: $V=(ab^2)/2$, where a is the longest diameter and b is the shortest diameter of the tumor (18). Blood was withdrawn by a heart puncture with a heparin-containing syringe.

Plasma troponin measurement. Plasma concentrations of cardiac troponin I (cTnI) were determined using a highsensitivity rat cardiac troponinI- ELISA (Life Diagnostics, Inc., West Chester, PA, USA). The results are expressed as ng/ml.

Echocardiographic assessment of cardiac physiological alterations. Two-dimensional B-mode and anatomical M-mode imaging were performed using the ultrasound imaging system Vevo 770 High-Resolution In Vivo Imaging System (VisualSonics, Toronto, ON, Canada). M-Mode images were acquired and used for the calculation of left ventricle (LV) function using calculation procedures provided by the manufacturer. The rats were anesthetized with 2% isoflurane/oxygen using a facemask during the whole procedure. All hair was removed from the chest using a chemical hair remover (Nair; Carter-Horner, Mississauga, Ontario, Canada). With the scanhead 716 and on B-mode, short-axis imaging was carried out to mainly visualize LV. M-Mode was used to obtain anatomically correct LV measurements, including LV posterior wall (LVPW) thickness, LV volume (LV Vol), LV ejection fraction (LVEF), and LV fractional shortening (LVFS). Data analysis was performed offline using a customized version of Vevo 770 Analytic Software.

Statistical analysis. Differences between means were considered significant at $p<0.05$, using unpaired *t*-tests of variance (StatView II; SAS Institute, Inc., Cary, NC, USA). All data were expressed as the mean±standard error of the mean (SEM).

Results

Body weight change and tumor size. The body weight change, which is a good indicator of the general health status of laboratory animals, is presented in Figure 1. The naïve controls gained an average of 15 g per week and at the end of the study (35 days after tumor implantation) had gained an average of 75 g. The untreated control tumor-bearing rats gained an average of 10g per week until day 21 after tumor implantation. However, at this time-point (day 21 after tumor implantation) all rats in the untreated tumor-bearing control group had to be sacrificed due to the enormous tumor burden. The rats treated with metronomic DOX lost significant body weight due to the suppressed food intake as early as day 7 after the start of the treatment and, due to their poor physical condition, were sacrificed at day 21. The rats from the metronomic CPA group maintained their body weight during the whole treatment period.

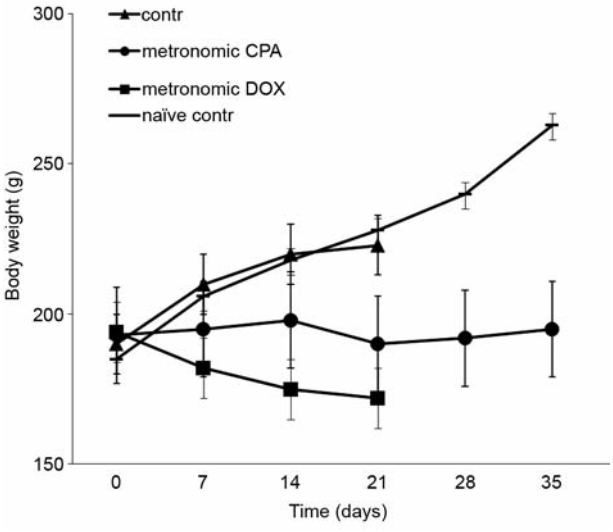

Figure 1. *Changes of body weight during the treatment of Mat B III mammary adenocarcinoma of rats using metronomic DOX and CPA. Data represent the mean±SEM of body weight.*

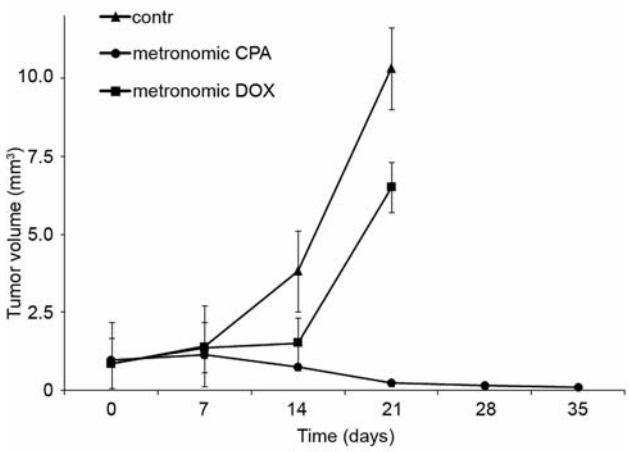

Figure 2. *Effect of metronomic DOX and CPA on Mat B III-induced mammary tumors of rats. Data represent the mean±SEM of tumor volume.*

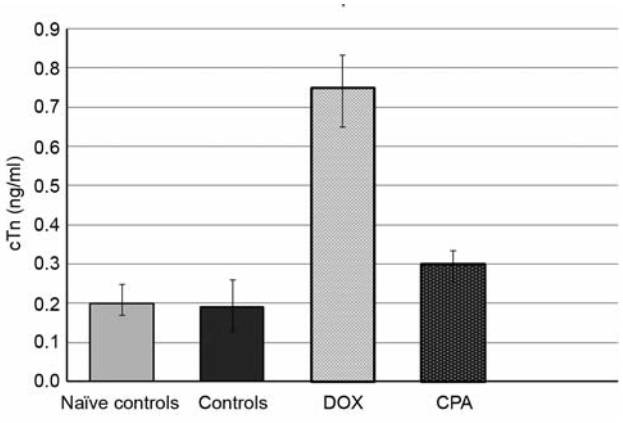

Figure 3. *Plasma levels of cTnI at sacrifice (two weeks after the completion of the treatment) of rats with Mat B III mammary adenocarcinoma treated with metronomic DOX and CPA. Data represent the mean±SEM of plasma cTnI. *p<0.05.*

The results for tumor growth (Figure 2) showed that metronomic CPA treatment was able to totally eradicate the tumors in all rats by day 21 after tumor implantation (Figure 2). Although at a slower rate, the tumors in the rats from DOX-treated group continued to grow.

Plasma troponin. Troponin I is a sensitive and specific marker of myocardial injury. Cardiac troponin I (cTnI) degradation has been noted in the stunned myocardium of rodents after ischemia and reperfusion and is a proposed mechanism for the decreased LV contractility in post-ischemic hearts (35). In this study, cTnI ELISA was used to measure cTnI in the plasma collected at sacrifice. The results showed more than a three-fold increase in the plasma levels of cTnI in the DOX-treated group in comparison with the untreated controls (Figure 3). There was no difference in cTnI plasma levels of rats with untreated tumors *versus* naïve controls (data not shown). The concentration of cTnI in the plasma of rats treated with CPA was significantly elevated in comparison with the untreated controls, but not as high as in the DOX-treated rats.

Echocardiographic assessment of cardiac physiological alterations. Heart rate was similar among the groups and was not affected significantly by tumor presence or by CPA and DOX treatment (range, 260-325 beats per minute).

At two weeks after completion of the treatment, the average LVPW end-diastolic thickness was decreased in both DOX- and CPA-treated rats in comparison to the baseline, although the reduction was significant only for DOX-treated rats (Table I). LV Vol was increased in both CPA- and DOX-treated groups of rats in comparison to the baseline, but the increase was statistically significant only for the DOX-treated group. The average reduction of LVEF in the DOX-treated group was more than 10% (average 12% decrease), in comparison to the baseline and the same effect was detected for LVFS. The metronomic CPA also reduced LVEF but the reduction was only 2%.

Discussion

It has been stated that the cardiotoxicity and efficacy of a chemotherapeutic drug depends on many different factors, including the dose, schedule of delivery, route of administration and combination with other drugs, as well as

Table I. *LV echocardiographic parameters at baseline and at two weeks after the completion of metronomic treatments with DOX and CPA of tumor-bearing rats.*

	LVPWd (mm)	LVPWs (mm)	LV Vol d (μl)	LV Vol s (μl)	LVEF (%)	LVFS (%)
Baseline	1.90±0.2	2.72±0.3	190.8±7.4	29.9±2.9	84.6±2.4	56.9±1.3
DOX	1.85±0.3*	2.62±0.4	195.7±6.1*	31.4±2.3	74.4±3.1*	50.1±1.8*
CPA	1.88±0.2	2.68±0.3	192.4±8.1	30.2±2.1	82.9±2.6*	55.8±1.7*

LVPWd/s, LV posterior wall diastolic/systolic; LV Vol d/s, LV volume diastolic/systolic; LVEF, LV ejection fraction; LVFS, LV fractional shortening. Data are presented as mean±SEM. *Significantly different from baseline, $p<0.05$.

the individual patient (36). The advantages of metronomic chemotherapy include reduced acute toxicities such as cardiotoxicity, high-grade myelosuppression, vomiting, nausea and mucositis (37, 38), as well as good antitumor activity. In fact, several recent reports have found that metronomic chemotherapy with some cytotoxic drugs for treatment of several types of cancer, such as breast cancer (39, 40), hormone-refractory prostate cancer (41, 42), sarcoma (43), melanoma (44) and ovarian cancer (45), may be better tolerated and have greater antitumor effects.

The aim of the present study was to examine the cardiotoxicity and efficacy of metronomic administration of CPA and DOX, two of the most commonly used chemotherapeutic agents. The results showed that CPA was able to target Mat B III-induced breast tumors efficiently, while DOX chemotherapy was not effective, due to its toxicity. Metronomic CPA treatment eradicated the tumors and was associated with lack of cardiotoxicity. In contrast, despite its initial efficiency in reducing tumor growth, metronomic DOX treatment was associated with significant body weight loss and more than 10% reduction of LVEF. A decline of LVEF by more than 10% in patients was suggested as a criterion for suspending the treatment (2, 46).

Abnormalities of LV structure and function are common in DOX-treated survivors (48). Chronic anthracycline-induced cardiomyopathy usually presents within a year of treatment. It may persist or progress even after discontinuation of anthracycline therapy and may evolve into a chronic dilated cardiomyopathy in adult patients and restrictive cardiomyopathy in pediatric patients (14). Late-onset chronic progressive anthracycline cardiotoxicity causes ventricular dysfunction (50), heart failure and arrhythmias (16) years to decades after chemotherapy has been completed. This suggests that patients who have received anthracycline chemotherapy and survived their cancer may have undetected increases in morbidity and mortality due to cardiotoxicity. Some studies (52) have suggested that DOX may be better tolerated if infused continuously for longer periods or in combination with cardioprotective agents. None of these approaches have had more than limited success and, despite the risks, DOX is still included in most chemotherapeutic regimens because of its broad-spectrum antitumor activity. In the breast cancer model of the present study, the metronomic administration was not able to prevent the severe cardiotoxicity of DOX.

Acute decompensating cardiomyopathy induced by CPA is usually irreversible. Lee *et al.* (53) investigated the clinical course and the outcome of therapy with CPA of thirteen patients and found that eight out of nine patients died of acute fatal restrictive cardiomyopathy with unresponsive hypotension, whereas three out of four patients who survived the initial episode died of subacute congestive heart failure. A significant number of experimental and clinical reports have indicated the efficiency and reduced side-effects of metronomic CPA. For example, Rozados *et al.* (54) treated mice with experimental lymphomas and sarcomas with low-dose CPA on a 3-time-weekly schedule and observed 100% eradication of cancer with no primary recurrence. At the same time, this regimen was devoid of BW loss, hematologic, cardiac, hepatic and renal toxicity. Man *et al.* (32) reported that metronomic administration of daily low-dose CPA is safe and efficacious in immune-compromised SCID mice inoculated with different cell types. Their model showed that metronomic CPA chemotherapy regimens may target multiple tumor-associated cell types. Zhao *et al.* (55) investigated the effect of continuous low-dose therapy with CPA on Dunning prostate R3327-AT1 rat tumors and found a significantly delayed tumor growth. Several clinical studies have shown a reduced toxicity and higher efficiency of metronomic CPA (38, 56). Recently published results from a phase I/II trial of metronomic chemotherapy with daily CPA, dalteparin and methotrexate in women with metastatic breast cancer showed that the therapy was safe, well tolerated and clinically active in metastatic breast cancer (58).

The results of the present study showed that metronomic CPA not only eradicated experimental Mat B III-induced breast rat tumors, but was also associated with a reduced cardiotoxicity, suggesting that the success of a metronomic chemotherapy in terms of both efficacy and toxicity, strongly depends on the target, the class and the schedule of administration of the anticancer agent.

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