Many efficacious cancer treatments cause significant cardiac morbidity, yet biomarkers or functional indices of early damage, which would allow monitoring and intervention, are lacking. In this study, we have utilized a rat model of progressive doxorubicin (DOX)-induced cardiomyopathy, applying multiple approaches, including cardiac magnetic resonance imaging (MRI), to provide the most comprehensive characterization to date of the timecourse of serological, pathological, and functional events underlying this toxicity. Hannover Wistar rats were dosed with 1.25 mg/kg DOX weekly for 8 weeks followed by a 4 week off-dosing “recovery” period. Electron microscopy of the myocardium revealed subcellular degeneration and marked mitochondrial changes after a single dose. Histopathological analysis revealed progressive cardiomyocyte degeneration, hypertrophy/cytomegaly, and extensive vacuolation after two doses. Extensive replacement fibrosis (quantified by Sirius red staining) developed during the off-dosing period. Functional indices assessed by cardiac MRI (including left ventricular ejection fraction [LVEF], cardiac output, and E/A ratio) declined progressively, reaching statistical significance after two doses and culminating in “clinical” LV dysfunction by 12 weeks. Significant increases in peak myocardial contrast enhancement and serological cardiac troponin I (cTnI) emerged after eight doses, importantly preceding the LVEF decline to <50%. Troponin I levels positively correlated with delayed and peak gadolinium contrast enhancement, histopathological grading, and diastolic dysfunction. In summary, subcellular cardiomyocyte degeneration was the earliest marker, followed by progressive functional decline and histopathological manifestations. Myocardial contrast enhancement and elevations in cTnI occurred later. However, all indices predated “clinical” LV dysfunction and thus warrant further evaluation as predictive biomarkers.

Abbreviations

A  late ventricular filling velocity
AIC  anthracycline-induced cardiotoxicity
CMR  cardiac magnetic resonance imaging
cTnI  cardiac troponin I
CO  cardiac output
DOX  doxorubicin
E  early ventricular filling velocity
Gd-DTPA  gadolinium (III) diethyltriaminepentaacetic acid
IU  international units
LVEF  left ventricular ejection fraction
SV  stroke volume

As cancer survival rates improve, the development of treatment-induced cardiotoxicity is becoming increasingly clinically relevant (Gianni et al., 2008; Trachtenberg et al., 2011). Childhood survivors of cancer are seven times more likely to die from cardiac causes and 15 times more likely to develop cardiac failure than their contemporaries (Mertens et al., 2001; Oeffinger et al., 2006). Anthracyclines are the leading cause of cardiac morbidity in cancer survivors (Mulrooney et al., 2009) but they are still highly effective anticancer agents, particularly against hematological malignancies and breast cancer. Subclinical impairment of left ventricular (LV) function has been reported in >50% of patients in 10 years following anthracycline therapy.
with clinical symptoms occurring in 5–19% of patients (Migrino et al., 2008). The incidence of anthracycline-induced cardiotoxicity (AIC) increases with cumulative dose (Lefrak et al., 1973), and development of AIC can ultimately lead to heart failure. Further to this, any pre-existing cardiac dysfunction may preclude treatment with anthracyclines, resulting in suboptimal treatment and poorer prognosis (Cardinale et al., 2006; Hershman et al., 2008).

Doxorubicin (DOX) is understood to have a multimodal cardiotoxic action, including impairment of mitochondrial metabolism, disruption of calcium modulation, and direct DNA damage (Montaigne et al., 2012; Takahashi et al., 1998; Zhang et al., 2012). DOX has recently been shown to impair mitochondrial respiration in permeabilized myocardial fibers (Montaigne et al., 2010). This occurs concomitantly with a reduction in hydrogenated nicotinamide adenine dinucleotide (NADH) redox state, mitochondrial membrane potential, and glucose uptake. DOX causes cardiomyocyte apoptosis by a reactive-oxygen species (ROS)-dependent mechanism (Wang et al., 2002) involving redox cycling of the drug by mitochondrial complex 1 and NADH dehydrogenase. In contrast to the ROS-dependent mechanism in cardiomyocytes, DOX-induced apoptosis in tumor cells is predominantly p53-dependent (Wang et al., 2004). Inhibition of guanylate cyclase has previously been demonstrated in rats exposed to a single DOX dose (Lehotay et al., 1979), and patient heart biopsies have revealed early decreases in cyclic guanosine monophosphate (GMP) and a rise in the cyclic AMP:cyclic GMP ratio at both 4 and 24 h postdose (Unverferth et al., 1983). These observations indicate that the underlying cardiotoxicity mechanisms appear to be similar in rats and humans, and therefore the rat is an appropriate model species for obtaining translationally relevant data regarding the development and progression of DOX-induced cardiotoxicity.

Despite our mechanistic knowledge, certain gaps still exist in our understanding of DOX-induced cardiotoxicity, particularly a detailed definition of the steps leading to severe impairment of cardiac contractility in mammals, including any early functional changes. Advances in cardiac imaging have started to reveal early subclinical damage, such as diastolic dysfunction, in patients receiving chemotherapy (Cochet et al., 2011; Stoodley et al., 2012). Further, cardiac magnetic resonance imaging (CMR) enables objective characterization of myocardial tissue that allows subtle cardiac damage and pathologies such as fibrosis and inflammation to be detected (Christian et al., 2012), which could also be applied to detect DOX-induced lesions. Myocardial degeneration is recognized as a feature of DOX-induced cardiotoxicity and this cell death is associated with release of cardiac troponin (cTn) into the circulation. However, it is unclear (1) exactly when cTn is released with respect to pathological lesion development, (2) when myocardial degeneration occurs in relation to cardiac contractile impairment, and (3) if these events are coupled. Ultimately, such information could enable the earlier detection of chemotherapy-induced subclinical cardiomyopathy in the clinic and support preclinical strategies in the safety assessment of novel drug candidates.

In light of this, we have undertaken a rigorous, integrated characterization of the serological, pathological, and functional events associated with the development of chronic DOX-induced cardiotoxicity in rat. In order to achieve this, we first aimed to identify a DOX dosing regimen in rat that resulted in a chronic, rather than acute, cardiomyopathy, consistent with clinical presentation (Elliott, 2006). Previously, in some studies, large single bolus doses of DOX, or multiple doses over 1–2 weeks, have been administered to rodents to evoke acute cardiotoxicity (Arola et al., 2000; Czarnecki, 1984; Takasaye et al., 2004). Our aim was to establish a more progressive cardiomyopathy, through weekly dosing over a 2-month period followed by a 1-month follow-up, in order to provide the optimal opportunity to gain insight into the chronology of events underpinning this toxicity. Herein, using state-of-the-art approaches including CMR, we provide the most comprehensive characterization to date of the timecourse and interrelationships between the serological, pathological, and functional events underlying DOX cardiotoxicity in rat.

**MATERIALS AND METHODS**

**Study outline.** All studies utilized 10-week-old male Hanover Wistar rats (Harlan, UK) housed two animals per cage. The animals were allocated to treatment groups using randomization according to body weight. Animal identification, conditions of housing, acclimatization, environment, diet, and water were in accordance with the standard AstraZeneca operating procedures in line with directive 2010/63/EU of the European Parliament. Clinical observations and food consumption were monitored daily and body weights were monitored biweekly. Treated rats (n = 6 for pilot study and n = 8 for longitudinal imaging study) were administered with a single intravenous bolus dose of DOX hydrochloride salt 1.25 mg/kg (equivalent to approximately 50 mg/m² in humans) or vehicle alone (0.9% (wt/vol) sterile sodium chloride) via tail vein once per week for 8 weeks followed by an off-dose “recovery” period of 4 weeks. For the pilot study, imaging was performed at week 12 under terminal anesthesia prior to necropsy. For the longitudinal imaging study, CMR was performed under recovery anesthesia on days 1 (baseline), 15, 29, 43, 57, and under terminal anesthesia on day 78. Anesthesia was performed by inhalation of 3% isofluorane in medical air. Blood samples were taken at baseline and prior to each dose. Where imaging and dosing days corresponded, contrast was given through the same cannula. For the satellite pathology study, treated rats (n = 6 per group) and control rats (n = 3 per group) were culled at each scanning timepoint for histological analysis of the heart. The animals were treated, sampled and terminated by taking one animal from each treatment group sequentially, and then repeating this until all the animals were processed. The animal samples were analyzed by processing all
of the samples from the same treatment group to avoid the risk of cross-contamination.

**CMR imaging.** All imaging experiments were carried out at AstraZeneca, Alderley Park using a 4.7 T Bruker system with Avance III electronics (Bruker BioSpin GmbH, Germany). Contrast enhanced sequences were performed using gadolinium (III) diethyltriaminepentaacetic acid (Gd-DTPA) contrast media. For full details of imaging sequences, see online supplementary methods section.

**Serological biomarker analysis.** Blood was collected into lithium heparin tubes and plasma prepared by centrifugation at 3000 rpm for 10 min at 4°C. Plasma was stored at −80°C until analysis. Cardiac troponin I (cTnI) was assessed using a human ultrasensitive troponin I assay kit (Siemens, Camberley, UK) validated for use in the rat on the Advia Centaur CP automated analyser (Siemens). Analysis was conducted as per manufacturer’s instructions with Liquichek Cardiac Markers Plus Control LT (Bio-Rad Laboratories, Irvine, CA) quality control material.

**Tissue processing and histopathological assessment.** Pathological assessment was performed on rats taken from a satellite pathology group that were treated in exactly the same way as the longitudinal imaging group animals. Rats were sacrificed on days 8, 15, 29, 43, 57, and 78, allowing direct comparison of the cardiac pathology with imaging and plasma biomarkers. The hearts were carefully removed and processed as follows: The tip of the right atrium was taken from all animals, fixed in 2.5% glutaraldehyde and stored for processing and examination by electron microscopy (EM). For histopathological examination, 4 μm tissue sections were cut and air dried onto strongly adhesive slides (Leica, Germany) and then deparaffinized in xyline (2 × 3 min) and rehydrated. Tissue sections were stained with hematoxylin and eosin (H&E) and examined by light microscopy. Fibrosis assessment was performed by staining for collagen using Picosirius red (Junqueira et al., 1979; Puchtler et al., 1973) and quantified using the “Color Deconvolution v9” algorithm to quantify the percentage of strongly staining collagen present in the myocardium. For full details of above methods, see online supplementary methods section.

**Pathological scoring of DOX-induced cardiac lesions.** Pathological scoring of the H&E stained cardiac sections from individual animals was performed according to a grading system developed by the authors based on Billingham and Bristow’s criteria with refinement to incorporate extracellular damage (Billingham and Bristow, 1984). Note that, unless stated, the pathological descriptors of each ascending severity grade are additional features to those of the lower grades: grade 1, multi-focal intracellular vacuolation of cardiomyocytes (minimal disruption of tissue architecture); grade 2, multi-focal neutrophilic and lym-phoplasmacytic inflammation (chronic active myocarditis) and endothelial hypertrophy; grade 3, multi-focal cardiomyocyte degeneration/apoptosis/necrosis; grade 4, multi-focal displacement of cardiomyocyte alignment/architecture with replacement macrophage/fibroblast infiltration; grade 5, diffuse grade 4 with multi-focal cardiomyocyte hypertrophy/cytomegaly; grade 6, diffuse grade 4 with diffuse cardiomyocyte hypertrophy/cytomegaly; grade 7, multi-focal intercellular vacuolation (edema) and fibroblast proliferation with collagen deposition (fibrosis); grade 8, diffuse grade 7; grade 9, atrial dilatation; grade 10, intra-atrial thrombosis.

**Statistical methods.** All statistical analyses were performed using Microsoft Excel 2007. Intergroup comparisons have been made using either a one- or two-tailed Student’s t-test as appropriate and 95% confidence intervals calculated. Non-paired t-tests were used for intergroup comparisons. Correlations between biomarkers used in the same animals were made using Pearson’s product moment correlation coefficient and the t-distribution of this coefficient was used to infer significance of the correlation.

**RESULTS**

**Development of a Rat Model of Chronic DOX-Induced Cardiomyopathy**

To develop a rat model of chronic DOX-induced cardiomyopathy, we initially performed a 12-week pilot study, applying CMR approaches to assess cardiovascular function in Hanover Wistar rats after DOX administration at 1.25 or 2.5 mg/kg/week for 8 weeks followed by a 4-week off-dosing period. Due to intolerance, animals in the 2.5 mg/kg dosec group had to be dose-reduced to 1.75 mg/kg during week 4 and the majority of these animals were terminated for welfare reasons between weeks 5 and 8. Adverse signs included respiratory effects (increased depth and decreased rate), cold extremities, decreased activity, pale skin, hunched posture, pilo-erection, tip toe gait, reduced food consumption, and unacceptable loss of body weight (up to 19% in total). A dose of 1.25 mg/kg/week was generally well tolerated, with a reduction in body weight gain and slightly reduced food consumption (compared with controls), throughout the 12-week study. Of note, 8 weeks of the 1.25 mg/kg dosing regimen equates (mg/kg basis) to the maximum recommended life time DOX exposure in humans (Bovelli et al., 2010; Swain et al., 2003).

CMR assessment of the 1.25 mg/kg DOX group at 12 weeks demonstrated a statistically significant reduction (treated vs. control p = 0.0008) in left ventricular ejection fraction (LVEF) to a mean value of <50% in the treated group. Having established a DOX-induced cardiomyopathy rat model, we next sought to use this to investigate the functional, structural, and pathological events encompassing the development of DOX-induced cardiotoxicity in more detail and to understand the ex-
Longitudinal Assessment of DOX-Induced Cardiac Dysfunction Using CMR

The functional indices derived from the volumetric analysis revealed significant differences between DOX treated rats and control animals over time. Multi-slice cine CMR revealed statistically significant decreases in multiple indices of cardiac function (Fig. 1). LVEF was significantly lower in DOX-treated animals relative to controls at the first post-DOX assessment (day 15), after only two doses (Fig. 1A). LVEF decreased incrementally with each subsequent DOX dose and continued to decline further during the postdosing “rest” period (day 50 onward), indicating that the DOX schedule had triggered a continuous and irreversible functional deterioration. Despite the steady decline in cardiac contractility, the mean rat LVEF remained in what is considered to be the “clinically normal (human) range” (Murray et al., 2009). Further during the postdosing “rest” period (day 50 onward), indicating that the DOX schedule had triggered a continuous and irreversible functional deterioration.

The incremental decline observed in both cardiac output (CO) (Fig. 1B) and stroke volume (SV) (Fig. 1C) in the DOX-treated rats was consistent with the LVEF profile of these animals. Of note, in the control group, CO increased 13% between baseline and day 57 ($p = 0.018$) in line with growth of the rats, whereas the DOX group did not significantly change weight post the second dose (day 15). There were no significant changes in heart rate (HR), left ventricular mass (LVM), and end diastolic volume (EDV), suggesting that these parameters did not deteriorate with DOX treatment (data not shown). Diastolic function was estimated by measuring the E/A ratio. This fell incrementally with time and also showed a statistically significant difference between DOX-treated and control rats from day 15 ($p = 0.02$) onward. The E/A ratios were 0.85 and 1.14 (DOX-treated vs. controls) by the end of study (Fig. 1D) representing significant diastolic dysfunction in the DOX group (Nagueh et al., 2009).

Elevations in Gd-DTPA Myocardial Enhancement and Serum cTnI are Biomarkers of DOX-Induced Cardiac Injury

Gd-DTPA is used to assess late-enhancing cardiac lesions, such as infarcts, in clinical practice (Saraste et al., 2008). Dynamic CMR imaging acquired during the infusion of Gd-DTPA demonstrated statistically significant increases in both peak myocardial enhancement (from day 57, Fig. 2A) and delayed myocardial enhancement (from day 43, Fig. 2B) in the DOX-treated group. Interestingly, when cTnI levels were assessed in serum samples taken from the same rats, circulating cTnI levels significantly increased during the nondosing period (days 50–78, Fig. 2C). Further, it was clear that the cTnI profile mirrored the myocardial enhancement profiles (Figs. 2A–C), suggesting that these biomarkers may be reporting a common event, for example, the onset of myocardial degeneration. In support of this, we found statistically significant correlations between serum cTnI levels and the degree of peak myocardial enhancement (day 57, $r = 0.71$, $p = 0.02$) and delayed myocardial enhancement (day 78, $r = 0.85$, $p = 0.03$). Little change was seen in peak enhancement in control animals throughout the time-course study and cTnI levels remained unchanged (Figs. 2A–C).

DOX-Induced Pathological Effects in the Heart

At the final timepoint (day 79), wet weights of the hearts at necropsy revealed a significant reduction in mean heart weight for DOX-treated rats relative to controls (0.94 g vs. 1.10 g, $p = 0.041$). However, when adjusted for body weight, there was no significant difference in heart/body weight ratios between DOX and control groups at any timepoint. H&E stained sections of the hearts were examined and assessed by a board qualified veterinary pathologist. Vehicle-treated control animals exhibited heart morphology equivalent to normal rats, with no discernible microscopic lesion(s) throughout the 78-day experiment (Figs. 3A, 3G, and 3H). DOX-treated rats exhibited normal cardiac morphology 1 week after a single dose (day 8, Fig. 3B). However, after two doses (day 15), some animals had developed multi-focal intracellular vacuolation of cardiomyocytes (Fig. 3C) in the atria (three/six rats) and ventricles (two/six rats). Of note, these early (grade 1) intracellular changes were associated with minimal disruption of the overall tissue architecture (Fig. 3C). After four DOX doses (day 29), (grade 2) multi-focal neutrophilic and lymphoplasmacytic inflammation (chronic active myocarditis) and endothelial hypertrophy was prevalent in the atria and to a lesser extent, the ventricles. By day 43 (after six doses), multi-focal cardiomyocyte degeneration (apoptosis and/or necrosis) was evident in the atria of rats (grade 3, Fig. 3D), in some cases, extending to displacement of cardiomyocyte alignment with replacement inflammatory cell infiltration (grade 4) and cardiomyocyte cytomegaly (grade 5). Although the ventricular pathology was consistently less severe than the atrial observations, the nature of the findings was consistent and progressively worsened over time (Figs. 3G and 3H). By day 57 (eight doses), intercellular vacuolation (edema) and fibroblast proliferation with collagen deposition (fibrosis) was observed in the atria (Fig. 3E). During the off-dosing period (days 50–78), the cardiac lesion progressively worsened to ultimately include atrial dilatation in all animals. Diffuse cardiomyocyte hypertrophy/cytomegaly was observed (Fig. 3E) along with extensive intra- and intercellular vacuolation (Figs. 3E and 3F). Diffuse disruption of the overall tissue architecture was clearly evident (Fig. 3F). Regions of hemorrhage were frequently observed (Fig. 3F) and in some hearts, large intra-atrial thrombi were also present at day 78.

Interestingly, we found a correlation between the degree of diastolic dysfunction (E/A ratio) in rats at the end of the study (day 78) and both the atrial and ventricular pathology severity scores ($r = 0.70$, $p = 0.03$; $r = 0.73$, $p = 0.03$, respectively).
FIG. 1. Detection of cardiovascular functional impairment by CMR during the development of chronic DOX-induced cardiomyopathy. Rats \((n = 8)\) were dosed once weekly with DOX (1.25 mg/kg, dashed line) or vehicle (solid line) for 8 weeks followed by a 4-week off-dosing period. Ejection fraction (EF) (A), cardiac output (B), stroke volume (C), and E/A ratio (D) were assessed by cardiac MR at baseline and throughout the 12-week study as described in the Materials and Methods section. Error bars represent 95% confidence intervals. Star symbols represent statistical significance (*\(p < 0.05\), **\(p < 0.01\), and ***\(p < 0.001\)).

suggesting that these indices may be coupled with respect to the progression of the cardiotoxicity. We also observed a correlation between cTnI levels and both atrial and ventricular pathology grade on day 43 (\(r = 0.94, p = 0.002; r = 0.76, p = 0.04\), respectively) and a correlation between cTnI and ventricular pathology only on day 57 (\(r = 0.80, p = 0.03\)).

Subcellular Effects of Chronic DOX Treatment on Rat Cardiomyocytes

Subcellular changes induced by DOX treatment were assessed by transmission electron microscopy (TEM) performed on rat heart sections. Cardiomyocytes from control rat atrium displayed a typically uniform myofibrillar, mitochondrial, and Z-line presentation with a highly regular Z-line orientation and a normal microvascular profile (Fig. 4A). Overall, there was a time-dependent progression in cardiomyocyte structural degeneration associated with DOX treatment (Figs. 4B–F; timepoints day 8 to day 78, respectively). A key feature of the subcellular lesion was longitudinal myofibrillar splitting and Z-line distortion, which was marked in individual cardiomyocytes even 1 week after a single DOX dose (day 8, Fig. 4B), and when no changes were evident at the cellular/tissue level in the H&E stained sections (Fig. 3B).

Of particular prominence were early mitochondrial changes, characterized by swelling, membrane distortion, dense-body deposition, and general loss of structural integrity (Figs. 4B–D). These were accompanied by areas of membrane blebbing, lysosomal prominence, and marked intracellular vacuolation (Figs. 4D and 4E). Interestingly, earlier changes were often more prominent in individual cells, with adjoining cells frequently displaying changes of lesser severity. At later timepoints, individual cellular changes assumed confluence and groups of cells were similarly affected. This corresponded with areas of focally extensive degeneration as seen by light microscopy (Fig. 3). Although there was a general preservation of intercalated disk configuration, scattered cells appeared to have lost all muscle fiber content, and contained myriad clear vacuoles surrounded by partially intact basal laminae (Fig. 4F). After chronic treatment (days 57 and 78), cardiomyocytes displayed marked microvascular congestion or intravascular “sludging” (Figs. 4E and 4F).
Elevations in serum cTnI and Gd-DTPA myocardial enhancement are biomarkers of DOX-induced cardiac injury. Rats (n = 8) were dosed once weekly with DOX (1.25 mg/kg, dashed line) or vehicle (solid line) for 8 weeks followed by a 4-week off-dosing period. Peak (A) and delayed (B) Gd-DTPA myocardial enhancement were assessed by CMR at baseline and throughout the 12-week study as described in the Materials and Methods section. Serum cTnI (C) was assessed using a Siemens human ultrasensitive cTnI assay kit. Error bars represent 95% confidence intervals. Star symbols represent statistical significance (*p < 0.05, **p < 0.01, and ***p < 0.001).

**FIG. 2.** Elevation in serum cTnI and Gd-DTPA myocardial enhancement are biomarkers of DOX-induced cardiac injury. Rats (n = 8) were dosed once weekly with DOX (1.25 mg/kg, dashed line) or vehicle (solid line) for 8 weeks followed by a 4-week off-dosing period. Peak (A) and delayed (B) Gd-DTPA myocardial enhancement were assessed by CMR at baseline and throughout the 12-week study as described in the Materials and Methods section. Serum cTnI (C) was assessed using a Siemens human ultrasensitive cTnI assay kit. Error bars represent 95% confidence intervals. Star symbols represent statistical significance (*p < 0.05, **p < 0.01, and ***p < 0.001).

**Replacement Fibrosis Development upon Chronic Doxorubicin Dosing**

Based on the observed fibroblast infiltration and apparent collagen deposition, particularly in the atria, observed in the H&E stained sections (Fig. 3E), we decided to examine collagen deposition within the myocardium in more detail. Picosirius red (SR) staining for collagen highlighted a fine trabecular fibrous stroma between cardiomyocytes within both the atria and ventricles of control animals (Fig. 5A). Digital quantification of the SR staining in control animals revealed no significant changes in collagen content over the duration of the study (data not shown). However, the normal collagen content of the atria was greater than that of the ventricle (Fig. 5C). DOX-treated rat hearts exhibited thickened and expanded intercellular fibrous stroma (scarring) in the ventricles, and more extensively in the atria, at the end of the study on day 78 (Fig. 5B). Quantification confirmed this and revealed statistically significant increases in the collagen content of DOX-treated rat heart ventricles and atria (Fig. 5C). At earlier timepoints, DOX-treated rat hearts did not show quantifiably significant collagen deposition, indicating that this is primarily a late event in the development of chronic DOX-induced cardiotoxicity.

**DISCUSSION**

In the study described here, chronic dosing of rats with DOX led to the development of a progressive cardiotoxicity and a number of key findings: (1) When measured by the sensitive modality of CMR, significant multi-parameter functional decline was one of the earliest, rather than latest features of DOX-induced cardiotoxicity. (2) Steady state circulating cTnI and myocardial Gd-enhancement elevations formed part of the progression of DOX-induced cardiotoxicity and preceded the LV dysfunction, but these are not predictive biomarkers of the onset LVEF decline per se. (3) Subcellular myofibrillar and mitochondrial degeneration coincided with (and is likely to be mechanistically linked to) early DOX-induced functional impairment and this preceded any overt cardiomyocyte degeneration.

Multi-slice cine CMR revealed statistically significant DOX-induced decreases in indices of both systolic (LVEF, CO, and SV) and diastolic (E/A ratio) cardiac function simultaneously from day 15. Pseudoimprovement was seen in diastolic function from day 15 to day 29, reflective of atrial damage preceding ventricular changes. This perceived improvement may result from poor atrial compliance rising intra-atrial pressures and driving passive filling. However, from day 29, a steep decline in diastolic function is seen as the damage progresses and atrial contraction is lost. In the clinical setting, diastolic dysfunction has been reported to precede systolic (Lee et al., 1987; Marchandise et al., 1989; Tjeerdsema et al., 1999) but our study shows a decline in all indices from day 15. The functional indices we measured continued to decrease incrementally with each subsequent DOX dose and declined further during the postdosing...
FIG. 3. Pathological characterization and severity scoring of the DOX-induced cardiac injury. Scale bar on each panel represents 100 μm. (A) Control animal (day 78) showing typical myocardial architecture. The pericardial surface (solid arrow), the endocardium of the atrial lumen filled with red blood cells (dashed arrow), and the normal arrangement of cardiomyocytes (zig-zag arrow) are highlighted. (B) DOX-treated rat heart section (day 8) exhibiting normal myocardial architecture. The pericardial surface (solid arrow), the endocardium of the atrial lumen (dashed arrow) and the normal arrangement of cardiomyocytes (zig-zag arrow) are all highlighted. (C) DOX-treated rat atrial section (day 15) exhibiting grade 1 pathology. The pericardial surface is shown (solid arrow) and multifocal intracellular vacuolation of cardiomyocytes highlighted (dashed arrows). (D) DOX-treated rat atrial section (day 43) exhibiting grade 3 pathology. Minimal disruption of overall architecture observed. Atrial lumen illustrated (triangle). Multi-focal and well-demarcated intracellular vacuolation of cardiomyocytes (dashed arrows); lymphoplasmacytic inflammation (solid arrow) and a degenerate cardiomyocyte (bent arrow) are highlighted. (E) DOX-treated rat atrial section (day 57) exhibiting grade 7 pathology. Diffuse cardiomyocyte hypertrophy/cytomegaly (solid arrows), intercellular vacuolation (edema; dashed arrows) and fibroblast proliferation with collagen deposition (fibrosis; zig-zag arrow) are highlighted. (F) DOX-treated rat atrial section (day 78) exhibiting grade 7 pathology. Diffuse cardiomyocyte hypertrophy/cytomegaly (solid arrows), intra- and intercellular vacuolation (dashed arrows) and diffuse disruption of architecture. Hemorrhage (triangle) and fibrosis (bent arrow) are also highlighted. Grading of ventricular (G) and atrial (H) cardiac pathology in rats over the timecourse of DOX treatment according to the system devised by the authors and described in the Materials and Methods section. The control data shown represents combined D57 and D78 vehicle-only control groups (n = 6), the DOX-treated groups are represented at each timepoint (n = 6). Error bars represent 95% confidence intervals. Star symbols represent statistical significance (*p < 0.05, **p < 0.01, and ***p < 0.001).
Fig. 4. Subcellular morphological characterization of DOX-induced changes in rat cardiomyocytes. Micrographs were prepared of representative cardiomyocytes. (A) Control rat (day 8) ×5000; (B) DOX-treated (day 8) ×4000; (C) DOX-treated (day 29) ×3000; (D) DOX-treated (day 48) ×4000; (E and F) DOX-treated (day 72) ×3000. Highlighted features—regularity of myofibrillar Z-line orientation (solid arrows) and the unadulterated microvascular profile (dashed arrow) within control rat cardiomyocyte (A). Longitudinal myofibrillar splitting and Z-line distortion (solid arrows) in cardiomyocytes from DOX-treated rats (B). Mitochondrial changes, characterized by swelling, membrane distortion, dense-body deposition, and general loss of structural integrity (bent arrows B, C, and D). DOX-treated cardiomyocytes contained large numbers of vacuoles (zig-zag arrows D and E) and some cells appeared to have lost all muscle fiber content (F) and contained a myriad clear vacuoles surrounded by partially-intact basal laminae (dashed arrows, F). Terminal samples displayed microvascular congestion (intravascular “sludging,” triangles, E and F).

“recovery” period (day 50 onward), indicating that, by the end of dosing, the DOX schedule had triggered a continuous and irreversible functional deterioration.

Despite the steady decline in cardiac contractility, the mean rat LVEF remained in what is considered to be the “clinically normal (human) range” (McMurray et al., 2012) until day 78 when it dropped to <50% (mean 49.2%, Fig. 1A), a level clinically indicative of left ventricular dysfunction. Although there were no premature deaths in the current study, in a previously published rat study dosing for 10 weeks with a higher dose of DOX (2.5 mg/kg), mortality significantly increased in the follow-up period (weeks 12–14) (Schwartz et al., 1998), providing further evidence of the progressive/irreversible nature of the lesion. Also consistent with our rodent findings, a significant decline in mean patient LVEF (vs. baseline) has been observed upon cessation of DOX therapy, which progressively worsens over the subsequent 12 months (Cardinale et al., 2006; Dodos et al., 2008).

The significant functional contractile decline we observed at day 15, after two DOX doses, coincided with low level (grade 1) multi-focal vacuolation in the hearts of some of the treated rats, and more notably, profound subcellular structural changes in cardiomyocytes as assessed by EM (from day 8) including longitudinal myofibrillar splitting, Z-line distortion and marked mitochondrial changes. Consistent with this, disruption of normal cardiomyocyte cytoskeleton, cross-striations, and a profound alteration in desmin localization (an intermediated filament protein required for contractile element anchorage) has previously
FIG. 5. Chronic DOX treatment is associated with replacement fibrosis in rat hearts. Fibrosis assessment was performed using Picosirius red staining for the presence of collagen and quantifying the percentage of myocardium with strong staining as described in the Materials and Methods section. The left ventricle and left atrium were considered as separate regions of interest and were quantified independently. (A) Control rat (day 78) atrioventricular junction. Picosirius red highlights the fine trebecular fibrous stroma found between cardiomyocytes within the atria (solid arrow) and ventricles (dashed arrow). Chambers contain residual red blood cells (triangle). (B) DOX-treated rat (day 78) atrioventricular junction. Sirius red highlights the thickened and expanded intercellular fibrous stroma (scaring) within the atria (solid arrow) and ventricles (dashed arrow). Vacuolation (cell loss) is clearly evident (bent arrow). (C) Box-plot showing a significant increase in Picosirius red staining between control (n = 3) and DOX-treated (n = 6) rat left ventricles (LV, p = 0.01) and left atria (LA, p = 0.026). Whiskers represent the upper and lower quartiles.

been observed after acute DOX treatment in rats (Arola et al., 2000). It is highly likely that the early functional decline we observed is a consequence of the observed DOX-induced damage to the subcellular contractile machinery, either directly or via defective protein synthesis in response to DNA damage. These subcellular DOX-induced features are highly consistent with previous findings in humans, particularly, the extensive myofibrillar degeneration and swelling to mitochondria and the sarcoplasmic reticulum (Lefrak et al., 1973). Interestingly, human cardiac biopsies examined post first dose of DOX have revealed mitochondrial swelling within the first 24 h (Unverferth et al., 1983).

At much later timepoints (after day 43), dynamic CMR imaging acquired during the infusion of Gd-DTPA demonstrated statistically significant increases in both peak and delayed myocardial enhancement, which mirrored steady state cTnI elevations. Further to this, significant correlations were found between myocardial enhancement and cTnI levels, suggesting that these biomarkers may be reporting a common event in chronically DOX-exposed hearts, most likely the onset of myocardial degeneration. Consistent with this, from day 43 (after six doses) onward, multi-focal cardiomyocyte degeneration (apoptosis and/or necrosis) became evident, particularly in the atria of rats. As described, we also observed correlations between cTnI levels and cardiac pathology grade on days 43 and 57. In a previous study of anthracycline-induced cardiomyopathy in rat, elevated serum cTnT correlated with a pathological “cardiomyopathy score,” which encompassed the extent of cytoplasmic vacuolation and myofibrillar loss (Herman et al., 1999). There is strong clinical evidence linking cTn elevation with cardiac ischemia (acute coronary syndromes) in man, which is thought to be due to myocardial necrosis from infarcted tissue (Antman et al., 1997). It is becoming evident that cTn can rise in other types of cardiac injury and our study supports this as cTnI was raised in the presence of the diffuse cardiac injury induced by DOX (Lauer et al., 1997).

Myocyte necrosis is characterized by cellular swelling, leading to uncontrolled leakage of cellular contents and an associated inflammatory response and appears to dominate (over apoptosis) in response to chronic DOX dosing (Arola et al., 2000). cTnT and cTnI are both highly sensitive and specific for cardiac injury (Jaffe et al., 2000, 2006). Although the mechanism(s) of myocardial degeneration induced by DOX is still not fully understood, it seems to be widely accepted that serum cTn elevations are a direct consequence of the cardiomyocyte degenerative process (Koh et al., 2004). However, whether cTn is solely released from necrotic cardiomyocytes or if it can be liberated as part of an apoptotic process, or even in response to reversible damage, all remain points of debate (Adamacova et al., 2005; Jaffe and Wu, 2012). As the majority of the cTn pool is complexed to the myocyte contractile machinery, it is possible that even mild-to-moderate cTn release may represent irreversible cardiac injury (Jaffe et al., 2000). Assuming loss of cardiomyocyte membrane integrity always precedes cTn re-
lease, early or acute cTn elevation is thought to derive from the smaller (~5%) soluble cytosolic cTn pool, which can be rapidly released after myocyte damage. In contrast, the sustained cTn elevation we observed likely results from breakdown of the contractile organelles, for example, through oxidative damage to the sarcolemma together with loss of cardiomyocyte membrane integrity via necrosis (Mair, 1997).

In the present study, we focused on the development of progressive cardiotoxicity and looked for significantly sustained cTnI elevations, or high steady-state levels, i.e., raised cTnI one week after the previous dose. These sustained elevations were only observed from week 6 (day 43) onward and reached significance at day 57 onward, 1 week after the last of the eight doses. The magnitude of the cTn elevations (0.02–0.03 ng/ml) is consistent with those previously observed in both rats and humans after anthracyclines (Herman et al., 1999; Ottinger et al., 1997). In the absence of continuous cardiac damage, raised serum cTn levels will quickly return to baseline after a few days. In drug-induced cardiotoxicity, the diagnostic window is likely to be drug-dependent and the mechanism of cardiac injury may be a crucial factor in the mode of myofibril loss and cTn release (Adamcova et al., 2005). Our data demonstrate that a combination of continuous myofibrillar system damage combined with sustained cTn release are key underlying features of the developing stages of progressive DOX toxicity.

In our rodent study, the cTnI elevation persisted for the 4 weeks between cessation of dosing and necropsy. The mean cTnI value increased to a maximum on day 78 and importantly, the LVEF decline mirrored this finding. By the end, the rodents had undergone a profound cardiac functional decline consistent with the onset of heart failure, in line with other preclinical studies (Bertinchanch, 2003).

In the clinic, the timing and extent of cTnI elevation is prognostic with respect chemotherapy-associated cardiac risk. Patients without cTnI elevations had no change in LVEF on follow-up (1 month to 3 years) (Cardinale et al., 2004). Marked “early” elevations in cTnI measured within 72 h of a high-dose chemotherapy cycle were associated with an enhanced risk of subsequent systolic dysfunction and clinical heart failure on follow-up. However, the cardiovascular risk is greatest for those patients exhibiting a persistent or “late” cTnI elevation 1 month after ending chemotherapy (Cardinale et al., 2000, 2002, 2004).

Like the functional indices, the pathological severity score increased with the total cumulative dose of DOX and the lesion continued to progress after cessation of dosing. The microscopic pathological features observed at later timepoints by us and others (Migrino et al., 2008; Schwartz et al., 1998) are highly consistent with those observed in postmortem human cardiac tissue after DOX-induced heart failure, namely disruption of the normal cellular architecture, cytoplasmic vacuolation, cellular loss, replacement fibrosis and even ventricular and atrial thrombi (Lefrak et al., 1973). Importantly of note, the chronic dosing regimen chosen for this translational model reflects that of clinical practice (Mettler et al., 1977).

In a study by Lightfoot et al., the effects of 10 weekly doses of DOX (1.5 or 2.5 mg/kg) on CMR parameters in Hannover Wistar rats was assessed (Lightfoot et al., 2010). In contrast to the present study, authors did not observe a significant early drop in LVEF that incrementally declined with repeat dosing. A significant LVEF decline was observed at the week 4 timepoint for the high dose group but not at the lower dose or at other timepoints. The authors showed that individual animals (from either DOX dose group) that experienced a “primary event” (deterioration in LVEF >10% of baseline, absolute LVEF <65% or unanticipated death) also exhibited myocellular degeneration. An increase in delayed myocardial Gd-enhancement was found to precede a “primary event” and was therefore a predictive biomarker of LV dysfunction (Lightfoot et al., 2010).

Considering our data in the context of the Lightfoot et al. primary event criteria, we observed a significant LVEF decline to <10% of baseline in the DOX-treated rats from day 29 (~12%), absolute LVEF was <65% from day 57 (58%) and no unexpected deaths occurred. We observed significant elevations in delayed and peak Gd-enhancement from days 43 and 57, respectively. Therefore, we conclude that the predictivity of Gd-enhancement for LV dysfunction ultimately depends upon the dysfunction criteria set. Nonetheless, it is clear from our study that significant LV impairment commences several weeks prior to elevations in myocardial Gd-enhancement, overt myocyte degeneration and the release of cTnI. Highly significant elevations in both peak and delayed Gd-enhancement were evident in the DOX group at day 57 when mean LVEF was 57% (<27% of baseline). The continued decline in cardiac function in the “recovery period” day 50 indicates that the cardiotoxicity was already irreversible when robust elevations in the myocardial Gd signal was observed. In the future, it would be informative to explore if the progressive cardiotoxicity can be halted or even reversed by ceasing after fewer doses, e.g., prior to the overt pathological changes and Gd-enhancement and cTnI elevations.

Our histological findings suggest that irreversibility could result, in part from diffuse fibrosis and remodeling. Lightfoot et al. did not observe an increase in fibrosis (staining and quantification was not performed) (Lightfoot et al., 2010). Our findings show that delayed Gd-enhancement and fibrotic changes occur concurrently following drug cessation, advocating delayed Gd-enhancement as a potential surrogate of cardiac remodeling. Focal myocardial enhancement is known to equate to areas of necrosis (peak enhancement) and subsequently fibrosis (delayed enhancement) from acute ischemic damage and this correlates with troponin elevation (Selvanayagam and Porto, 2005). However, the role of diffuse global Gd-enhancement in the setting of progressive drug-induced cardiotoxicity has not yet been explored in the clinical setting.

In summary, for the first time we have comprehensively characterized the chronology and nature of not only the functional events but also the pathological and serological changes underlying progressive DOX-induced cardiotoxicity in a clinically relevant rodent model (Fig. 6). Using sensitive imaging tech-
Summary illustration of cardiac changes associated with the development of DOX-induced cardiomyopathy. Cartoon illustration showing the chronology of DOX-induced cardiac changes. Key functional parameters (EF, CO, SV, and E/A) declined significantly from baseline when assessed at day 15 (after two DOX doses) and continued to decline throughout the study. Subcellular (EM) changes were marked at day 8 (after a single DOX dose) and progressed thereafter. DOX-associated pathological changes at the tissue level were not evident at day 8 but developed from day 15. Significant elevations were observed in delayed Gd-enhancement (from day 43), in peak Gd-enhancement and cTnI (from day 57), consistent with the pathological onset of cardiomyocyte degeneration. Fibrosis development was a late event (significant at day 58), consistent with collagen deposition in regions of cardiomyocyte degeneration.

In order to better understand the injury, we have characterized myocardial signal intensity changes and correlated them with pathological events and circulatory changes. We are currently characterizing equivalent CMR and serological parameters in the clinical setting. It is hoped that these studies will help us to gain an understanding of the translational and patient-related aspects of DOX-induced cardiac injury, inform predictive biomarker research, and support the development of safer future medicines.

**SUPPLEMENTARY DATA**

Supplementary data are available online at [http://toxsci.oxfordjournals.org/](http://toxsci.oxfordjournals.org/).

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![Functional baseline](image1.png)

![Biomarker baseline](image2.png)
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