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Inhibition of chymotryptic-like standard proteasome activity exacerbates doxorubicin-induced cytotoxicity in primary cardiomyocytes

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ABSTRACT

The anthracycline doxorubicin (DOX) is a potent anticancer agent for multiple myeloma (MM). A major limitation of this drug is the induction of death in cardiomyocytes leading to heart failure. Here we report on the role of the ubiquitin-proteasome system (UPS) as a critical surveillance pathway for preservation of cell vitality counteracting DOX treatment. Since in addition to DOX also suppression of proteasome activity is a rational therapeutic strategy for MM, we examined how small molecular compounds with clinically relevant proteasome subunit specificity affect DOX cytotoxicity. We found that during DOX-treatment, the activity of the β5 standard proteasome subunit is crucial for limiting off-target cytotoxicity in primary cardiomyocytes. In contrast, we demonstrate that the β5 equivalent LMP7 of the immunoproteasome represents a safe target for subunit-specific inhibitors in DOX-exposed cardiomyocytes. Neither inhibition of LMP7 in primary cardiomyocytes nor genetic ablation of LMP7 in heart tissue influenced the development of DOX cardiotoxicity. Our results indicate that as compared to compounds like carfilzomib, which target both the β5 standard proteasome and the LMP7 immunoproteasome subunit, immunoproteasome-specific inhibitors with known anti-tumor capacity for MM cells might be advantageous for reducing cardiomyocyte death, when a combination therapy with DOX is envisaged.

1. Introduction

The efficacy of anthracycline (ANT) drugs like doxorubicin (DOX) is well proven for a variety of tumor entities. Given the role of topoisomerase II – the protein that is mainly affected by ANT (Zhang et al., 2012) – for cellular homeostasis, it is not surprising that toxicities associated with such drugs frequently occur. Particularly the induction of cardiotoxicity leading to functional impairment of the heart muscle is a common threatening off-target effect and often necessitates discontinuation of therapy. The prevalence of ANT-cardiotoxicity increases in a dose-dependent manner. Nevertheless, ANTs are potent anti-cancer agents and therefore much effort was put into the investigation of molecular aspects underlying ANT-induced cardiotoxicity. ANTs are known to cause DNA damage, inhibit transcription of specific gene programs as well as protein synthesis, and to induce cardiomyocyte apoptosis via a caspase-3-dependent mechanism (Sterba et al., 2013). Moreover, ANT-induced inhibition of topoisomerase II β leads to the formation of reactive oxygen species (ROS) and

Abbreviations: DOX, doxorubicin; MM, multiple myeloma; UPS, ubiquitin-proteasome system; ANT, anthracyclin; ROS, reactive oxygen species; SP, standard proteasome; IP, immunoproteasome; PI, proteasome inhibitor; CM, cardiomyocyte; ABP, activity-based probe.

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increases the amount of oxidant-damaged proteins in heart muscle cells (Zhang et al., 2012).

As a consequence of an elevated amount of damaged proteins, the cellular unfolded protein response is induced. The ubiquitin-proteasome system (UPS) is a major cellular protein degradation pathway, and UPS-dependent protein turnover is essential for maintaining protein homeostasis particularly during stress conditions (Ciechanover, 2005). Cellular proteins can be marked by a poly-ubiquitin chain, which in case of a K48-ubiquitin linkage formation targets these proteins for recognition by 26S proteasome complexes for unfolding and degradation of the respective protein (Chau et al., 1989). In addition to degrading misfolded and damaged proteins, the UPS also determines the availability of regulatory proteins that control transcription, cellular proliferation, inflammation, and antigen processing as well as apoptotic cell death (Kloetzel, 2001). The catalytic activity of the proteasome is restricted to three of the seven β-subunits all located within the 20S proteasome core complex. The standard proteasome (SP) with its catalytic subunits β1, β2, and β5 is expressed in all cells. SP is the predominant proteasome type found both in cardiomyocytes and in heart tissue (Jakel et al., 2009; Szalay et al., 2006). Among various different subtypes of 20S proteasome complexes, there is a second major isoform, the immunoproteasome (IP), which harbors the LMP2/β1i, Mecl-1/β2i and LMP7/β5i catalytic subunits (Aki et al., 1994). This isoform is preferentially expressed in cells of hematopoietic origin including MM and leukemia cells (de Bruin et al., 2015; Parlati et al., 2009). As shown by our group and others, the IP can also be found in heart muscle tissue of naive mice (Gomes et al., 2006; Jakel et al., 2009; Szalay et al., 2006) and can be upregulated in cardiomyocytes by cytokines (Opitz et al., 2011). IP abundance increases whenever cells are stressed (Kruger and Kloetzel, 2012). Consistent with the facilitated protein degradation capacity of the IP in comparison to its SP counterpart, IP formation is advantageous for maintaining protein homeostasis and preserving cell vitality under conditions of increased substrate supply to the UPS (Ebstein et al., 2013; Opitz et al., 2011; Seifert et al., 2010).

In the past decade, inhibition of proteasome activity by small molecular compounds like the boronate bortezomib has been a very successful approach for treatment of multiple myeloma (MM) (Richardson et al., 2003, 2005). The success of inhibiting proteasome activity by small molecular inhibitors for such hematologic malignancies is mostly attributed to suppression of proliferation and activation of apoptosis in cancer cells. Despite the high efficacy of proteasome inhibitors (PI) in MM patients, discontinuation of therapy is often necessary e.g. due to severe off-target effects. These circumstances supported the development of alternative compounds with different proteasome subunit specificity. Carfilzomib is such a potent subunit-specific PI that is known to exert profound anti-tumor activity in MM (Parlati et al., 2009; Stewart et al., 2015). This epoxyketone-based compound selectively and irreversibly binds to the β5 SP and LMP7 IP subunit (Parlati et al., 2009), thereby reducing the chymotryptic-like activity of the proteasome (Dick et al., 1998).

The majority of patients receiving such PI is often treated additionally with a combination of other chemotherapeutic drugs—including DOX. Although the safety concern of such combinatory treatment approaches is still a matter of debate, large clinical studies suggested that a combination of DOX and PI might deteriorate DOX-induced cardiotoxicity (Richardson et al., 2005; Siegel et al., 2013; Sophia et al., 2015). However, this issue has never been addressed experimentally at a molecular level. As the proteasome was suggested to act as a carrier of DOX into the nucleus of a cell (Kiyomiya et al., 1998), elucidation of a potential alteration of proteasome function by DOX has also become relevant. Over time, different groups have addressed this important question mostly with conflicting results on the impact of DOX-treatment on proteolytic activity by the proteasome complex (Ranek and Wang, 2009). Nevertheless, there is a consensus that the ubiquitination machinery – a critical rate-limiting step in proteasome-dependent proteolysis – becomes up-regulated in cardiomyocytes. As such, muscle-specific E3 ligases might facilitate heart muscle protein degradation and cardiomyocyte atrophy (Yamamoto et al., 2008), the latter being a hallmark of DOX cardiotoxicity.

In this study, we tackled the question whether UPS function might be affected by DOX and how the proteolytic capacity of defined proteasome subunits with known clinical impact for treatment of MM patients might influence the development of DOX cardiotoxicity. We used several different approved biochemical methods in a cellular model of primary cardiomyocytes to investigate proteasome function during DOX exposure. Since proteasome activity can be modulated at the subunit expression level and by different regulators, we studied proteasome composition and complex formation in response to DOX treatment. Further, we investigated the impact of new and highly promising selective PI specifically targeting the chymotryptic-like activity of the proteasome on DOX-induced cytotoxicity. Particularly, we discriminated the role of the SP β5 subunit and its IP equivalent LMP7 during the development of DOX cardiotoxicity.

2. Materials and methods

2.1. Animals

LMP7$^{-/-}$ mice on a C57BL/6 background (F7) originally generated by Fehling et al. (Fehling et al., 1994) were provided by Ulrich Steinhoff. Six weeks old male LMP7$^{-/-}$ and LMP7$^{+/+}$ mice (n = 12) were injected intraperitoneally (i.p.) with doxorubicin 25 mg/kg body weight. For echocardiography, mice were anesthetized with 1.5–2% isoflurane and kept warm on a heated platform. Temperature and ECG were continuously monitored. Cardiac function and morphology were assessed with a VisualSonics Vevo 2100 High-Frequency Imaging System with the use of a high-resolution (MS400; 38 MHz) transducer. Standard imaging planes, M-mode, Doppler, and functional calculations were obtained. The parasternal long-axis four-chamber view of the left ventricle (LV) was used to guide calculations of percentage fractional shortening, ventricular dimensions and volumes. M-mode echocardiographic images were recorded at the level of the papillary muscles from the parasternal short-axis view. Interventricular septum (IVS) and left ventricular posterior wall thickness (LVPW) were estimated at end diastole. An experienced reader blinded to treatment performed all measurements. All mice were sacrificed at day 4 after doxorubicin injection. This study was carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the German animal welfare act, which is based on the directive of the European parliament and of the council on the protection of animals used for scientific purposes. The protocol was approved by the Committee on the Ethics of Animal Experiments of Berlin State authorities. All efforts were made to minimize suffering.

2.2. Cell preparation, cultivation and transfection

C57BL/6 mice were anesthetized via isoflurane inhalation and euthanized by cervical dislocation on embryonic day 14. Embryonal hearts were isolated and incubated in 50 μl trypsin (0.05%, Biochrom, Germany) over night at 4 °C. On the following day, the hearts were incubated at 37 °C for 15 min. Then, embryonal cardiomyocytes (CM) were detached from the cell complex through careful suspending with 1 ml pre-warmed medium and seeded in 12-well-plates (Greiner bio-one, Austria) with 2.5×10^5