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#### Proteasomes are not a Target for Doxorubicin in Feline Injection-Site Sarcoma

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#### Summary

The potent anti-cancer agent doxorubicin (DOX) induces apoptosis of rapidly proliferating cells by inhibiting cellular proteasomes. The aim of the present study was to determine whether DOX modulates the level of expression and function of proteasomes in feline injection-site sarcoma (FISS). Tissue extracts from primary sarcoma lesions and the related healthy subcutis of 18 cats affected by FISS were investigated. Nine of these cats had received neoadjuvant DOX treatment and nine cats did not receive this therapy. There was enhanced proteasome expression in FISS, but this was not affected by administration of DOX. This finding may account for the low clinical effectiveness of DOX therapy in FISS and provides the rationale for developing new therapeutic protocols aimed at achieving better proteasomal inhibition in FISS and other tumours that respond poorly to DOX therapy.

## Introduction

Doxorubicin (DOX) is a potent anti-cancer agent of the anthracycline family that is widely used in the chemotherapeutic treatment of a variety of tumours. Despite its extensive clinical utilization, the exact mechanism by which DOX induces death of neoplastic cells remains controversial (Minotti *et al.*, 2004). To date, several different mechanisms have been proposed, including generation of free radicals, DNA binding and alkylation, intercalation into DNA, inhibition of helicase or topoisomerase II activities and direct membrane effects. Recent findings have suggested that proteasomes have an important role in modulating the anti-neoplastic activity of anthracyclines (Minotti *et al.*, 2004). Proteasomes are multicatalytic protease complexes involved in the degradation of proteins, including those implicated in the regulation of cell proliferation, survival and differentiation. The active form of the proteolytic complex is the 26S proteasome, formed by the association of the 19S regulatory cap with one or both ends of the 20S core particle. A special form of the proteasome is the immunoproteasome, which contains three novel catalytic b subunits that are induced by the immune modifier interferon (IFN)-γ (Goldberg *et al.*, 2002) and the proteasome activator PA28 (Cascio and Goldberg, 2005). Incorporation of these three subunits alters proteasome cleavage specificities and enhances the production of peptides able to associate with class I molecules of the major histocompatibility complex (MHC; Cascio *et al.*, 2001). Immunoproteasomes are found normally in lymphoid tissues (Cascio *et al.*, 2001; Goldberg *et al.*, 2002).

Proteasomes have also been shown to modulate the activity of anthracyclines (Minotti *et al.*, 2004) via at least two different mechanisms. Firstly, proteasomes act to translocate DOX from the cytoplasm into the nucleus via nuclear pores, in a process mediated by nuclear localization signals present in several a subunits of the 20S particle. Once in the nucleus, DOX dissociates from the proteasomes and binds to DNA (Kiyomiya *et al.*, 2001). Secondly, DOX binds to an allosteric site of the proteasome and acts as a noncompetitive inhibitor of proteasomal chymotrypsin-like activity (Figueiredo-Pereira *et al.*, 1996; Kiyomiya *et al.*, 2002a), which is the major proteasome activity and most likely the rate-limiting activity for protein breakdown *in vivo*. Therefore, the DOX-proteasome interaction may have a dual therapeutic role by increasing targeting of anthracycline to the nucleus (where it can damage DNA) and stabilizing pro-apoptotic factors (e.g. IkBa, p53, Bax) that are normally degraded by the ubiquitine-proteasome pathway (Hideshima *et al.*, 2003; Adams, 2004a; Cenci *et al.*, 2006).

Proteasomes also degrade proteins involved in the cell cycle, angiogenesis, adhesion, cytokine production and apoptosis (Glotzer *et al.*, 1991; Pagano *et al.*, 1995; Zhao *et al.*, 2000). Therefore, inhibition of proteasomes can affect tumour cell growth and lifespan via both direct and indirect mechanisms and proteasome inhibitors are emerging as powerful therapeutic tools in the management of many tumours. In particular, bortezomib (PS-341; Velcade<sup>TM</sup>; Millennium Pharmaceuticals, Cambridge, Massachusetts), a first-in-class proteasome inhibitor, is currently used for first-line treatment of multiple myeloma (Chauhan *et al.*, 2005). Although the specificity and exact mechanisms of action of individual proteasome inhibitors are still under investigation, the end result of inhibiting proteasome activity is apoptosis, providing increased sensitivity to standard chemotherapy and radiation therapy, and decreased resistance to these treatments (Adams, 2004b). High expression levels and enzymatic activities of immunoproteasomes have been demonstrated in feline injection-site sarcoma (FISS; Cerruti *et al.*, 2007), a spontaneously arising and highly locally invasive tumour of cats that is sometimes proposed as a model for the study of tumour biology in other species, including man (Vail and MacEwen, 2000; McNiel, 2001). The aim of the present study was to determine whether administration of standard clinical DOX treatment to cats with FISS might alter the expression level and activity of proteasomes *in vivo*.

#### Materials and Methods

#### Treatment Modalities

Eighteen cats with FISS were studied. Nine animals (group A) underwent wide-margin surgery without adjuvant treatment. The remaining animals (group B) were treated intravenously with 1 mg/kg body weight of DOX, every 3 weeks for four cycles, followed by en-bloc resection of the tumour 10 days after the second DOX administration.

#### Total Protein

Samples of sarcomas and healthy subcutis were collected from each animal during surgery. These were immediately placed on ice and homogenized in ice-cold extraction buffer (50 mM Trise HCl pH 7.5, 1 mM DTT, 250 mM sucrose, 5 mM MgCl<sub>2</sub>, 0.5 mM EDTA and 2 mM ATP) with an Ultraturax DIAX900 homogenizer (Heidolph Instruments, Kelheim, Germany) and centrifuged at 17,000g for 20 min at 4°C. The protein concentration in the resultant supernatants was determined using the QUICK STARTT<sup>M</sup> Bradford dye reagent (Bio-Rad Laboratories, Hercules, California) with a standard curve constructed by use of a dilution series of bovine serum albumin (BSA) of known concentrations. Samples were stored at -80°C.

#### Proteasome Activity Assays

Peptidase activity of proteasomes was assayed by monitoring the production of 7-amino-4-methylcou-marin (AMC) from fluorogenic peptides, as previously described (Santoni de Sio *et al.*, 2006, 2008). Briefly, Suc-LLVY-AMC (for chymotrypsin-like activity) and Z-YVAD-AMC (for caspase-like activity) (BACHEM, Bubendorf, Switzerland) were used at a final concentration of 100 mM in 20 mM Trise HCl pH 7.5, 1 mM ATP, 2 mM MgCl<sub>2</sub> and 0.2% BSA. Reactions were initiated by adding an aliquot of tissue extracts and the fluorescence of released AMC (excitation at 380 nm; emission at 460 nm) was monitored continuously at 37°C with a Carry Eclipse spectrofluorimeter (VARIAN, Palo Alto, California). Background activity (caused by non-proteasomal degradation) was determined by the addition of the proteasome inhibitor MG132 (BACHEM) at a final concentration of 10 mM.

#### Western Blotting Analysis

Western blotting analysis of the proteasomal and immunoproteasomal catalytic b subunits and PA28a/P in both sarcomas and normal subcutis tissue extracts was performed as previously described (Cascio *et al.*, 2002). Briefly, 60 mg of total proteins for X, Y, LMP2 and LMP7, and 40 mg for PA28a and b, were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (12% gel) and proteins were transferred to an Amersham Hybond-P membrane (GE Healthcare, Buckinghamshire, UK). Membranes were stained with Ponceau red before incubation with the primary antibody to confirm that similar amounts of protein had been transferred. The membrane was then incubated in blocking buffer (5% non-fat milk in 1 x Phosphate buffered saline; 0.1% Tween-20), followed by incubation with rabbit antisera against LMP2, LMP7, PA28a and PA28b (a kind gift of Dr. K. Tanaka, Tokyo Metropolitan Institute of Medical Science, Tokyo), X (a kind gift of Prof. A. L. Goldberg, Harvard Medical School, Boston), and mouse monoclonal anti-Y (a kind gift of Prof. A. L. Goldberg). Bound antibodies were 'visualized' by the enhanced chemiluminescence technique (Amersham EGLPlus; GE Healthcare) and densitometric analysis of bands was performed with a Versa-Doc 1000 Imaging System (Bio-Rad) and Quantity One software (Bio-Rad).

## Statistical Analysis

A Shapiro-Wilk test revealed that the densitometric data were not normally distributed (data not shown) and so a non-parametric Wilcoxon test was performed to establish whether protein levels differed significantly between sarcomas and related healthy subcutis. For this purpose, values were transformed into log10 (densitometric value + 1) and the ratio was ex-pressed as log10 (sarcoma/healthy subcutis protein expression). A Wilcoxon test was also used to establish whether chymotrypsin-like and caspase-like activities differed significantly between sarcomas and healthy subcutis. Finally, the Mann-Whitney U test was performed to determine whether proteasomal expression levels and activities differed significantly between DOX-treated and untreated patients. P < 0.05 was considered significant and P < 0.01 was considered highly significant. Data are presented graphically as box plots.

#### Results

#### Patients and Tissue Samples

Eighteen sarcoma lesions and matched normal subcutis were obtained from 10 female (one entire and nine neutered) and eight male (one entire and seven neutered) cats with an average age of 9  $\pm$  0.76 years (range 4-16 years; median 9 years), which had

undergone wide-margin surgery at the Veterinary Teaching Hospital of Turin University. Half of the animals (group B, Table 1) underwent surgery without any preoperative chemotherapy, while animals in the second group (group A, Table 1) received neoadjuvant DOX treatment according to a protocol used in countries where radiotherapy is not available (Barber *et al.*, 2000; Bregazzi *et al.*, 2001; Cohen *et al.*, 2001; Poirier *et al.*, 2002; Martano *et al.* 2005). Proteasomal subunit expression levels and catalytic activities in both groups were measured in tissue extracts from primary sarcoma lesions and healthy subcutis of the same animals (see below). Ten tumours were located in the subcutis of the interscapular region, three on the dorsal neck, two on the scapula, and three on the thoracic wall. Microscopical examination revealed that all lesions were fibrosarcomas. Tumour staging was based on the World Health Organization tumour-node-metastasis (TNM) classification system (Owen, 1980). Four tumours in each group were classified as  $T_2N_0M_0$  two as  $T_3N_0M_0$  and three as  $T_4N_0M_0$  (Table 1).

#### Proteasome Activity Assays

Chymotrypsin-like activity was nearly 25-fold higher in sarcomas than in the healthy subcutis of DOX-treated animals (Fig. 1, Table 2) and proteasomal caspase-like activity was 13-fold higher in sarcomas than in the matched healthy subcutis of treated cats (Fig. 1, Table 2). Similar up-regulation of proteasomal activity was found in tumour tissues compared with the healthy subcutis in the group of animals that were not treated with DOX (Table 2). The enhancement of proteasomal activity in neoplastic specimens did not appear to be modified by DOX administration (Table 2) and the ratios of chymotrypsin- and caspase-like activities between sarcomas and healthy subcutis did not differ significantly with DOX treatment (Table 3).

### Western Blotting Analysis

There was no difference in the expression levels of the two constitutive proteasomal catalytic b-subunits X and Y when neoplastic and corresponding healthy subcutis were compared (Fig. 2A, Table 3). In contrast, expression levels of the IFN- $\gamma$ -induced immunoproteasomal catalytic subunits LMP2 and LMP7 and PA28a and b were markedly enhanced in tumours compared with matched healthy tissues (Fig. 3A, Table 3). These differences were highly significant in both groups (A and B) (Fig. 3B, Table 3), but were not significantly correlated with either tumour stage or clinical outcome (data not shown). The ratios of expression of proteasome and immunoproteasome subunits between healthy and neoplastic tissues were not affected by DOX administration. The extent of up-regulation of proteasomal system components known to be induced by IFN- $\gamma$  (LMP2, 7, PA28a and b) in neoplastic tissues was very similar for both DOX-treated and untreated animals (Table 3), while the steady-state levels of the catalytic constitutive subunits (X and Y) were unchanged between sarcoma lesions and healthy subcutis regardless of DOX administration (Table 3).

	Number of cases	Age (years)*	Gender	Anatomical site	$T\mathcal{N}M$
Group A: DOX treated	9	$7.33 \pm 0.83$ (6)	Five female	Five interscapular	Four $T_2N_0M_0$
			Four male	One	Two $T_3N_0M_0$
				interscapular + neck	
				One neck	Three T <sub>4</sub> N <sub>0</sub> M <sub>0</sub>
				One scapula	
				One thorax	
Group B: Untreated	9	$10.67 \pm 1.04 \ (11)$	Five female	Four interscapular	Four $T_2N_0M_0$
			Four male	Two neck	$Two T_3N_0M_0$
				One scapula	Three T <sub>4</sub> N <sub>0</sub> M <sub>0</sub>
				Two thoras	

#### Table 1 Characteristics of sarcomas analyzed

TNM, tumour, node, metastasis classification system. Data are presented as the mean ± standard error of the mean (median).



Fig. 1. Increase in proteasomal chymotrypsin-like and caspase-like activities in tissue extracts from nine sarcomas compared with healthy subcutis (median 6.53 for chymotrypsin-like activity and 9.17 for caspase-like activity).

Table 2. Chymotrypsin-like and caspase-like activity ratios in untreated and treated cats

	Group A DOX treated	Group B Untreated	
Chymotrypsin-like activity	$24.83 \pm 9.21$ (6.53)	19.01 ± 7.98 (6.93)	
Caspase-like activity	$13.30 \pm 4.63 \ (9.17)$	15.37 ± 7.74 (7.60)	

Data are presented as the mean  $\pm$  standard error of the mean (median) of the ratios of related activity between sarcomas and healthy subcutis.

#### Discussion

In recent years, there has been an increased interest in understanding the role of the proteasomes in modulating the antitumor activity of anthracyclines (Minotti *et al.*, 2004). In particular, it has been reported that DOX acts as a reversible noncompetitive inhibitor of the chymotrypsin-like activity of proteasomes by binding to an allosteric site of the protease (Figueiredo-Pereira *et al.*, 1996). As a new class of drugs, proteasome inhibitors show powerful in-vitro and in-vivo anticancer activity, inducing apoptosis and increasing the sensitivity to radiotherapy and chemotherapy (Adams, 2004b). L1210 mouse lymphocytic leukaemia cells exposed to DOX accumulate ubiquitinated proteins and undergo a similar level of apoptosis as when exposed to inhibitors targeted at the catalytic site of the proteasome (Kiyomiya *et al.*, 2002a). Moreover, the intensity of the anticancer effects of different anthracyclines correlates with their binding affinity and inhibitory effects towards proteasomes (Kiyomiya *et al.*, 2002a,b).

The aim of the present study was to determine the effects of standard clinical DOX treatment on proteasome levels and activities in FISS (Hendrick *et al.*, 1992, 1994; Kass *et al.*, 1993; Hendrick and Brooks, 1994; Seguin, 2002). This spontaneously arising tumour is characterized by enhanced immunoproteasome expression levels and activities (Cerruti *et al.*, 2007), which makes it an attractive candidate for studying modifications induced by different drugs on proteasome homeostasis.

In the present investigation, tissue extracts from two groups (DOX treated and untreated) of cats affected by FISS were assessed for proteasomal subunit expression levels and catalytic activities. The two groups were similar in terms of their general characteristics and tumours (Table 1), so direct comparison of the proteasomal activities and expression levels in the presence or in the absence of DOX was possible. It was not possible to measurement proteasomal parameters in tissue from the same animal before and after DOX treatment, as this would have required two separate surgical procedures for each individual.

	A Sarcoma versus healthy subcutis (Wilcoxon test)*			B DOX treated versus untreated (Mann–Whitney test) <sup>†</sup>	
Chymotrypsin-like	Treated	P = 0.008	Highly significant	P = 0.546	Not significant
activity	Untreated	P = 0.008	Highly significant		
Caspase-like activity	Treated	P = 0.011	Significant	P = 0.863	Not significant
	Untreated	P = 0.008	Highly significant		
X	Treated	P = 0.214	Not significant	P = 0.931	Not significant
	Untreated	P = 0.173	Not significant		
Y	Treated	P = 0.260	Not significant	P = 0.161	Not significant
	Untreated	P = 0.314	Not significant		
LMP7	Treated	P = 0.008	Highly significant	P = 0.546	Not significant
	Untreated	P = 0.008	Highly significant		
LMP2	Treated	P = 0.008	Highly significant	P = 0.666	Not significant
	Untreated	P = 0.008	Highly significant		
PA28a	Treated	P = 0.008	Highly significant	P = 0.297	Not significant
	Untreated	P = 0.008	Highly significant		
ΡΑ28β	Treated	P = 0.008	Highly significant	P = 0.094	Not significant
	Untreated	P = 0.008	Highly significant		

Table 3 Differences in proteasome and immunoproteasome expression and activities between untreated and DOX-treated cats

P < 0.05 was considered statistically significant, whereas P < 0.01 was considered highly significant.

\*Wilcoxon test was performed to establish differences between proteasome expressions and activities in samples of sarcoma and healthy subcutis. †Mann-Whitney test was performed to establish differences between ratios (sarcoma/healthy subcutis) of untreated and DOX treated cats.

The enhanced levels of immunoproteasome expression and activities typical of FISS were not affected by the repeated administration of DOX. In fact, two catalytic b subunits of immunoproteasomes (LMP2 and 7) and two subunits (a and b) of the proteasome activator PA28 were expressed at higher levels in sarcomas than in the normal subcutis of cats with FISS that were treated with DOX. Moreover, the levels of two catalytic b subunits of constitutive proteasomes (X and Y) were approximately the same in sarcoma lesions and in matched healthy subcutis. As a functional counterpart of these results, tissue extracts from sarcomas showed enhanced proteasomal chymotrypsin-like activity, and to a lesser extent caspase-like activity, than healthy subcutis. Crucially, these differences in both enzymatic activity and immunoproteasomal subunit expression levels were very similar between DOX-treated and untreated animals. Furthermore, the same observation was made in respect of unchanged steady-state levels of the two catalytic subunits X and Y of the constitutive proteasome.



**Fig. 2.** Levels of proteasomal (X and Y) catalytic b subunits in sarcomas and healthy control subcutis. Nine specimens were analyzed. (A) Two representative western blot analyses for X and two for Y are shown. (B) Expression ratios of X and Y in sarcomas and healthy subcutis were calculated as described in Materials and Methods.



Fig. 3. Expression levels of immunoproteasomal catalytic b subunits (LMP7 and LMP2) and PA28 activators (a and b) in nine sarcomas and healthy control subcutis. (A) Two representative western blot analyses for LMP7 and LMP2 and two for PA28a and b are shown. (B) Box plots of the expression ratios of LMP7/2 and PA28a/b between sarcomas and matched healthy subcutis, determined as described in Materials and Methods.

These unexpected findings strongly suggest that the standard protocol of DOX administration to cats with FISS is unable to modulate intracellular proteasome functions in neoplastic fibroblasts. Eukaryotic cells are known to respond to a variety of stimuli that either decrease proteasome function or increase the demand for proteasomal function by enhancing proteasome biogenesis, thereby defining a proteasome stress response (Meiners *et al.*, 2003; London *et al.*, 2004; Lundgren *et al.*, 2005; Hanna and Finley, 2007). Although the mechanism based on the concerted transcriptional regulation of all proteasome genes still remains elusive in metazoans, it is nevertheless well established that mammalian cells up-regulate both the level and activity of proteasomes in response to pharmacological inhibition (Meiners *et al.*, 2003; Lee *et al.*, 2004; Lundgren *et al.*, 2005). Therefore, if DOX treatment in FISS displays any modulatory effects on proteasomes, it is very likely that the steady-state levels and activity of the enzyme should be affected following repeated administration of the drug. Since we could not take tissue samples from patients before wide-margin excision of the tumour, we could not assess proteasome levels or activities soon after DOX administration. However, it is unlikely that such measurements would be more informative, since in-vitro modulation of proteasomal steady-state levels requires at least 1 week (Wang *et al.*, 2000; Hong *et al.*, 2003; Fuchs *et al.*, 2008; Bianchi *et al.*, 2009), which probably reflects the low rates of synthesis of an extremely stable particle, with an estimated half-life of 2 weeks (Tanaka and Ichihara, 1989).

Importantly, the present findings may help explain the low therapeutic effectiveness of neoadjuvant DOX in animals affected by FISS (Martano *et al.*, 2005). In theory, higher levels of proteasomes might imply that the cancer cells have a requirement for enhanced proteolysis, perhaps to cope with cytotoxic stress related to dysregulated growth (Ma and Hendershot, 2004; Mathew *et al.*, 2007; Dang *et al.*, 2008). However, an alternative but more complex model has linked the sensitivity to proteasome inhibitors of normal and

neoplastic cells to a unique imbalance between reduced proteasomal activity and increased protein synthesis (Cenci *et al.*, 2006; Cascio *et al.*, 2008). The resulting overload of cellular capacity for degradation would render cells extremely vulnerable to proteasome inhibitors (Bianchi *et al.*, 2009).

In the context of FISS, it will be important to understand the molecular mechanisms underlying the low sensitivity of this tumour to anthracyclines and to develop more effective pharmacological strategies. The low sensitivity of FISS to the proteasomal inhibitory effects of DOX may be related to the predominant presence of immunoproteasomes in neoplastic fibroblasts. These alternative degradation particles show distinct enzymatic properties and therefore might be intrinsically more resistant to the inhibitory effects of DOX than normal proteasomes. Immunoproteasomes also display enhanced trypsin-like activity, which is not affected by anthracyclines and which might, at least partially, substitute for DOX-inhibited chymotrypsin-like activity. The results of the presents study suggest that it will be necessary to develop new anthracycline analogues that are more effective in inhibiting immunoproteasomes for the successful management of tumours such as FISS. It may also be possible to combine DOX with other drugs such as bortezomib, which is specifically designed to act as a proteasome inhibitor.

# **Conflict of Interest**

None of the authors has a financial or personal relationship with other persons or organizations that could inappropriately influence or bias the content of the paper.

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