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ORIGINAL ARTICLE

Ex vivo evaluation of imatinib mesylate for induction of cell death on canine neoplastic mast cells with mutations in c-Kit exon 11 via apoptosis

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Abstract Several studies of canine spontaneous mast cell tumours have described mutations in the c-kit proto-oncogene. These mutations produce a constitutively activated product and have been suggested to play a role in the malignant transformation of mast cells. We hypothesize that the selective tyrosine kinase inhibitor imatinib mesylate inhibits signal transduction and induces apoptosis when tested in cutaneous canine mast cell tumour samples positive for mutation in c-kit exon 11. Three-dimensional ex vivo cultures of canine grade II mast cell tumour treated with STI-571 at 48, 72, and 96 h and tested for signal transduction and apoptosis using appropriate assays were used. There was a progressive and significant increase in caspase-3 and TUNEL-positive mast cells compared to the untreated cultures. Additionally, a concurrent reduced expression of Ki67 and BCL-2 was observed. Furthermore, the treated cultures showed a marked reduction of Kit expression. Our results demonstrate that STI-571 induces Caspase-dependent apoptosis in a canine neoplastic mast cells possessing mutations in c-kit exon 11.

Keywords Apoptosis · STI-571 · Signal transduction · Immunohistochemistry · c-Kit

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Introduction

Mast cell tumours (MCT) are one of the most common cutaneous tumours in dogs, representing about 20 % of all cutaneous tumors. Most tumours occur in middle-aged dogs and among the more commonly recognized predisposed breeds are the boxer, pug, Boston terrier, bull terrier, weimaraner and Labrador retriever (Goldschmidt and Hendrick 2002). MCT have a highly variable biological behaviour, ranging from a benign biological behaviour to highly aggressive disease. Commonly reported metastatic sites include regional lymph nodes, liver, spleen, and bone marrow. MCT are frequently histologically quite diffuse and are thus difficult to control by local treatment; some of these may be resistant to systemic chemotherapy (Vail 1994). Cellular differentiation and thus, the histologic grade has been commonly used as a predictor of the biologic behaviour and prognosis of MCT (Patnaik et al. 1984). Poorly differentiated MCT may contain fewer cytoplasmic granules than well-differentiated tumours (Simoes and Schoning 1994). As previously stated, the biologic behaviour of canine cutaneous MCT is highly variable and unpredictable, and the ability to formulate an accurate prognosis is hampered by the large number of intermediate-grade (grade II) MCT. It has previously been demonstrated that canine mast cells (MCs) express CD117 (also known as proto-oncogene c-Kit or tyrosine-protein kinase Kit), an extracellular ligand binding domain for the hematopoietic stem cell factor (SCF) (Qiu et al. 1988). Mutations in the c-kit proto-oncogene, specifically deletions and internal tandem duplication (ITD) within c-kit exon 11, which correspond to the juxtamembrane region of Kit, have been implicated in the progression of canine cutaneous MCT. The ITD c-kit mutations are significantly associated with aberrant Kit protein localization in neoplastic MCs. Recently,

a study showed 3 patterns of Kit protein localization in canine MCT: peri-membranous, cytoplasmic stippling to focal, and diffuse cytoplasmic (Kiupel et al. 2004). Well-differentiated tumours weakly express Kit, whereas poorly differentiated tumours show high cytoplasmic-perinuclear expression of Kit (Kiupel et al. 2004).[5] The selective tyrosine kinase inhibitor imatinib mesylate (STI-571 or Gleevec®) has been licensed for the treatment of human chronic myeloid leukaemia (CML) and gastrointestinal stromal tumours (GIST) (Druker et al. 2001; Demetri et al. 2002). In human systemic mastocytosis, a rare disease characterized by an abnormal mast cell proliferation in blood and different organs, mutation of c-kit and platelet-derived growth factor receptor alpha are commonly identified and led to the development of targeted treatments, including STI-571 (Fain et al. 2005). Gleixner et al. (2007) showed growth inhibitory effects of four tyrosine kinase inhibitors, including imatinib, on C2 canine mast cell tumour, as well as primary neoplastic canine MCs. Understanding of the molecular mechanisms and effects of anticancer drugs in vivo is notoriously difficult, whereas the use of *ex vivo* tissue cultures collected from tumor samples during surgery represents an attractive and innovative approach. Three-dimensional (3D) tissue cultures seem to reflect more appropriately the in vivo patient situation than the more commonly used two-dimensional monolayer cell cultures, since the state of cell adhesion and expression of intercellular adhesion molecules in 3D culture decrease the chemosensitivity of neoplastic cells (Nakamura et al. 2003). Aims of this study were to examine the capability of STI-571 to induce apoptosis and to explore the pathway of apoptosis in an ex vivo culture system.

A dog with ITD-positive grade II MCT was enrolled for this study because only MCT possessing this mutation are appropriate for evaluation of STI-571 treatment on cell apoptosis. A grade II was used since the intermediate grade is highly unpredictable in terms of antitumor response and

Materials and methods

Ex vivo biopsies study

Twenty 8 mm diameter punch biopsies were obtained from a cutaneous, histologically confirmed, grade II MCT from a 10 year old, intact male Boxer after obtaining informed, written consent from the client. The biopsies were obtained from a large single ulcerate mass (15×12 cm) arising from the right shoulder consistent with a recurrence of a cutaneous nodule excised 1 year before and diagnosed as a grade I mast cell tumour. PCR analysis for c-kit exon 11 was performed as described by Isotani et al. (2008). A band of PCR products larger than the estimated size (190 bp) of wild-type c-kit amplification product was detected. The cutaneous tumour biopsies, deprived of hypodermis, were cultivated for 24 to 120 h in vitro. Briefly, tissue sample were taken from the operating room immediately after resection and placed in transport culture medium RPMI 1,640 medium (Sigma, St. Louis, USA) containing 10 %v/v heat-inactivated fetal bovine serum, 200 IU/mL penicillin (Gibco, Invitrogen, CA) and 200 mg/mL streptomycin (Gibco, Invitrogen, CA, USA). The specimens were subsequently placed into agarose-coated Petri plates (60 mmm X 15 mm) and incubated at 37 °C with 5 % CO_2. 7,5 ml of Leibovitz's L-15 containing 10 %v/v heat-inactivated fetal bovine serum, 200 IU/mL penicillin (Gibco, Invitrogen, CA), 200 mg/mL streptomycin (Gibco, Invitrogen, CA, USA) and STI-571 was added directly to five plates (treated group); the dose used for STI-571 was of 15,83 mg corresponding to the oral dose of 10 mg/kg as previously described (Isotani et al. 2008). The medium was changed daily. Five samples were maintained with the culture medium only (control group). The experiment was repeated three times to confirm the results.. The samples were examined at 24, 48, 72, 96, and 120 h by fixing the tissues in 10 % buffered formalin for 8 h. The tissues were then washed in sterile saline, dehydrated, paraffin embedded, placed on Superfrost Plus slides (Histoline, Milan, Italy) in 2 μm sections, dewaxed and stained with Haematoxylin and Eosin for microscopic examination. The extent of the neoplastic infiltrate at different times, in both treated and untreated samples, was scored as reported afterwards.

Immunohistochemistry

Paraffin embedded sections were used for immunohistochemical analyses. Rehydrated sections were treated for endogenous peroxidases neutralization with 3 % hydrogen peroxide (H_2O_2) for 5 min followed by rinsing for 5 min with distilled water. Antigen retrieval was achieved by incubating slides in an antigen retrieval solution in a steamer (Black & Decker, Towson, MD, USA) for 20 min. Nonspecific immunoglobulin binding was blocked by incubation of slides for 10 min with a protein-blocking agent (Dako, Carpinteria, CA, USA) before application of the primary antibody. The slides were then incubated overnight in a moist-chamber with the following primary antibodies: polyclonal rabbit anti-human CD117/c-kit (Diagnostic BioSystems, Pleasanton, CA, USA), polyclonal rabbit anti-human Ki67 (Santa Cruz, CA, USA), and rabbit anti-human Bcl-2 (Santa Cruz, CA, USA), all used at dilutions of 1:50. A streptavidin–immunoperoxidase staining procedure (Dako, Carpinteria, CA, USA) was used for immunolabeling. The immunoreaction was observed with 3,3′-diaminobenzidine or VIP substrate (Vector, Burlingame, UK). Sections were counterstained with Mayer's Haematoxylin. Positive immunohistochemical controls included archived MCT. Negative

immunohistochemical controls were represented by using histopathologically confirmed MCT treated identically as routine sections, with 20 min antigen retrieval and 10 min protein blocking, except for the fact that the overnight incubation with primary antibodies was replaced by an overnight incubation with buffer. Expression of cleaved caspase-3 in paraffin-embedded tissue sections was investigated using an affinity-purified rabbit polyclonal antibody (anti-active caspase-3, Promega Corporation, Madison, WI, USA) directed against a peptide from the p18 fragment of the cleaved human caspase-3. The antibody was first tested for its capacity to give positive signals on dog tissues, such as mesenteric lymph nodes. As described by Resendes et al. (2004), cleaved caspase-3 immunohistochemistry showed positive signals mainly in the nuclei of lymphocytes, and at a less extent in the cytoplasm, furthermore positive signals were more frequent in the germinal centres than in the interfollicular areas of lymph nodes. Lymphatic tissue was subsequently selected as the positive control for further tests. The primary antibody was replaced by phosphate buffered saline solution as a negative control.

TUNEL

In cutaneous sections, treatment-induced pro-apoptotic effect in the neoplastic cells was highlighted through a TUNEL colorimetric staining (DeadEnd, Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions.

Grading of neoplastic MCs, Kit, TUNEL, Caspase −3, Ki67, Bcl-2, and Kit–staining pattern

To score the tumour MCs and the Kit positive cells at different sample times in both treated and untreated biopsies, ten random fields were examined under a 40X. The total number of neoplastic MCs and c-Kit stained malignant MCs was recorded. The mean value obtained per histological section per time was analyzed.

To enumerate the TUNEL positive nuclei and cleaved Caspase-3 expression, tissues were graded according to the number of detected apoptotic cells by two independent blinded observers as follows:

0: none apoptotic signal; 1: low level of apoptotic signal (<5 %); 2: moderate level of apoptotic signal (5–10 %); 3: high level of apoptotic signal (10–20 %); 4: very high level of apoptotic signal (>20 %).

To evaluate the apoptotic rate, ten random fields of the sample were examined under a 40X objective. The number of positive MCs was normalized to the number of MCs per field and expressed as a percentage of these values.

Similarly Ki67, and Bcl-2 slides were visually scanned and scored: 0 (negative;< 5 % positive cells); 1 (sporadic; 5 % to 20 % positive cells); 2 (moderate; 20–50 %); 3 (diffuse; 50–75 %), and 4 (all; > 75 % cells).

Kit staining-pattern included three different categories, according to the classification reported by Kiupel et al. as follow. Category 1: membrane-associated staining, category 2: focal to stippled cytoplasmic staining with decreased membrane-associated staining and category 3: diffuse cytoplasmic staining. Malignant MCs from ten random 40X fields was assigned to one of the three categories.

For all parameters considered in this study, the cells at the edge of the tissue sections were excluded from evaluation to avoid possible artifactual staining.

Statistical analysis

Analysis of variance (2-way ANOVA) was applied to test differences between treatment and time of treatment (level of significance at $P<0.05$). Variation of parameters were investigated by Analysis of variance (ANOVA) to compare median value for parameters (Bcl-2, MCs number, Caspase-3, c-Kit, Ki67 and TUNEL) between the treated and untreated control biopsy. The level of statistical significance was set to $P<0.05$ throughout.

Results

Cultures treated with STI-571 showed a significant reduction of infiltrating neoplastic cells at the end of the observation period, as evidenced in Fig. 1a. In these samples, a progressive and significant increase in TUNEL positive MCs was observed at 72 h (Fig. 1b), and apoptotic activity peaked at 96 h post-treatment (PT) (Fig. 1b, Fig. 2). Similar results were obtained in sections stained for Caspase-3 (Fig. 1c). Incubation with STI-571 led to a progressive increase in Caspase-3 expression in ex vivo tumor tissue cultures with a peak at 72 h PT. Unlike untreated cultures, which did not stain with Caspase-3, the treated ones exhibited a strong staining as shown in Fig. 3. Malignant MCs of untreated controls showed an intact nuclear structure and the apoptosis rate of neoplastic cells remained constant throughout the experiment (Fig. 1b). Of note, untreated control cultures showed a higher apoptotic index than that expressed by routinely fixed MCT samples (data not shown). Ki67 nuclear staining was observed in untreated cultures up to 5 days (120 h), suggesting that neoplastic MCs maintain their capability to proliferate ex-vivo (Fig. 1d). In treated cultures, Ki67 (Fig. 1d) and BCL-2 (Fig.1e) expression decreased consistently with lack of expression for Bcl-2 72 h PT. A similar trend was observed also in Kit stained cells (Fig. 1f); positive cells were detected in similar percentage and pattern of stain in both treated and untreated biopsies, stopped 24 and 48 h PT

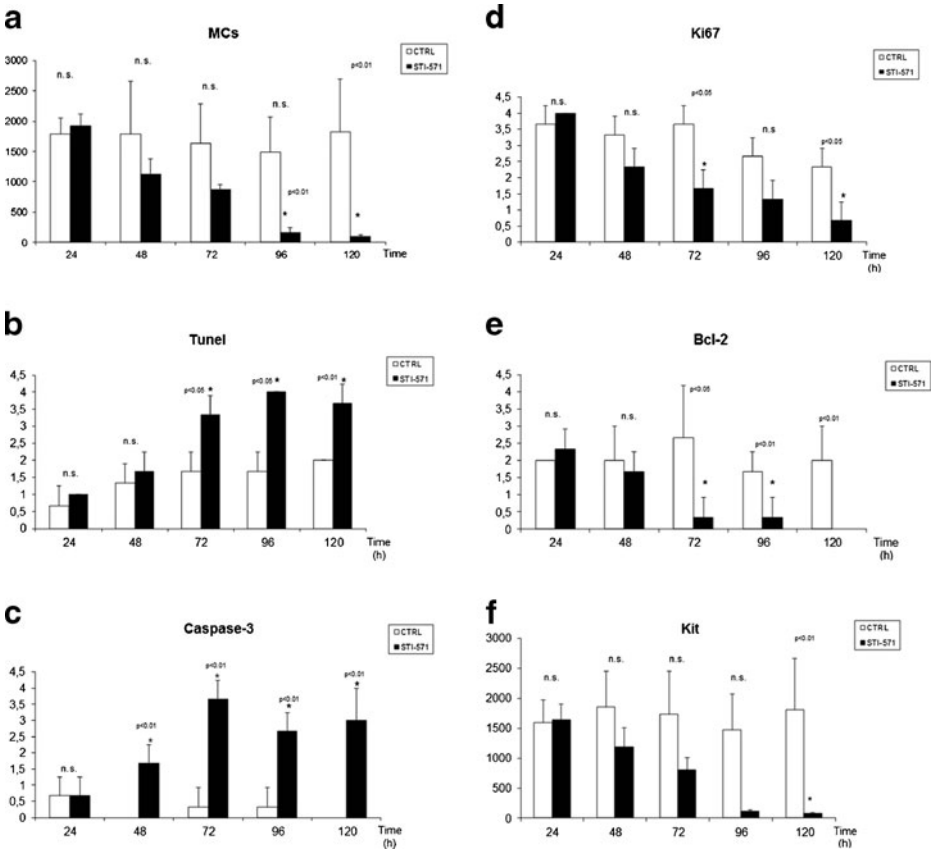

Fig. 1 Variations of parameters in biopsy before and after the STI-571 treatment (mean values ± standard deviations, $n=15$). Asterisks indicate significant differences between means of values within the same time treatment ($p<0.05$). ns=non-significant. **a** MCs; **b** TUNEL; **c** caspase-3; **d** Ki67; **e** BCL-2; **f** Kit

(Fig. 4). A dramatic reduction in Kit expression was observed in the treated samples stopped 96 h PT. (Fig. 5), with positive cells showing type III pattern. Untreated cultures showed a mixed pattern of staining, characterized by large clusters of neoplastic cells with "type II" pattern and at least 3 % of the neoplastic MCs with "type I" pattern. A trend toward large dimension and intense cytoplasmic Kit staining was noted in TUNEL and Caspase-positive MCs. Results of two-way analysis of variance for the parameters measured in biopsies, before and after the STI-571 treatment are summarized in Table 1.

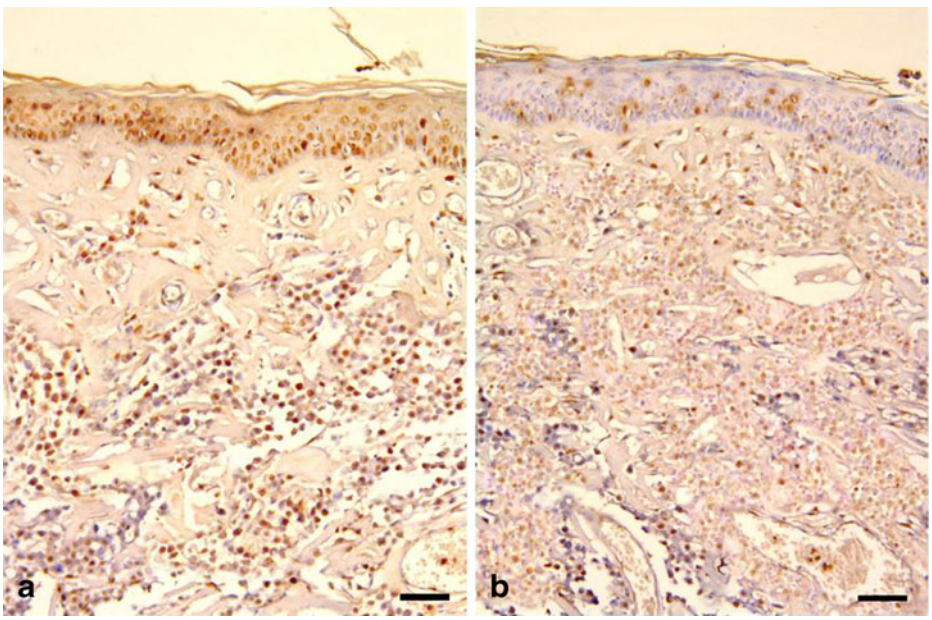

Fig. 2 TUNEL positive malignant MCs at 96 h. A: skin biopsy treated with STI-571. B: untreated biopsy (bar=25 μm)

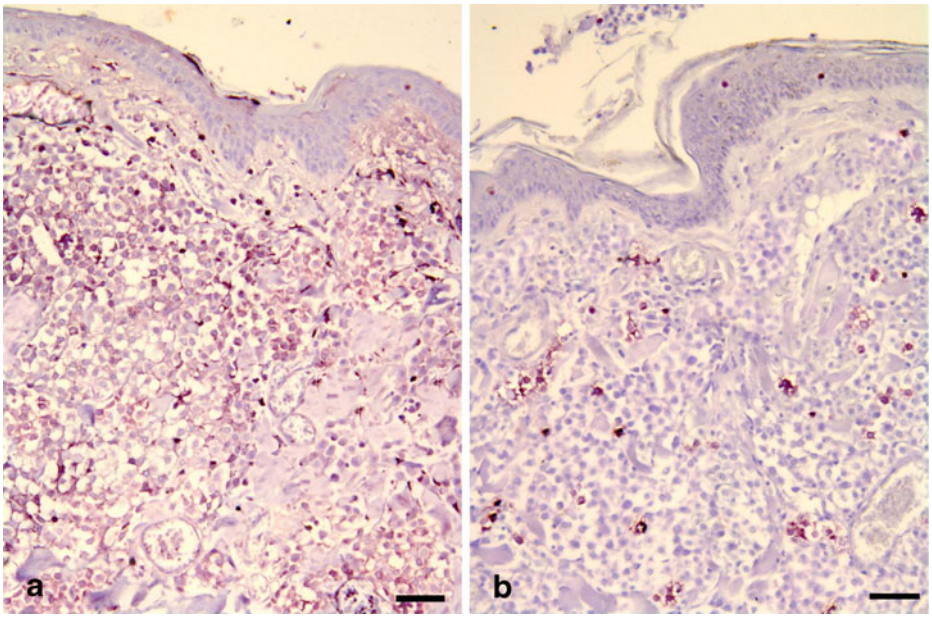

Fig. 3 Caspase-3-positive malignant MCs at 72 h. A: Skin biopsy treated with STI-571. B: untreated biopsy (bar=25 μm)

Discussion

This study demonstrates that the tyrosine kinase inhibitor STI-571 induces Caspase-dependent apoptosis in canine malignant MCs possessing c-Kit mutations. The use of a 3D ex vivo tissue culture confirmed this system as a useful tool for drug testing despite similar experiments done in MCT cell line with the mutation should be carried out in order to compare the results. The 3D architecture and the biological significance of the stromal cells are crucial for the function of normal organs and tumours, particularly in terms of interactional processes such as attachment, adhesion, spatial orientation, and impact of gravity, which all influence the biology of the involved cells (Nakamura et al. 2003). This finding is especially relevant in studies of therapeutic drug action in oncology, since adhesion and attachment have been shown to directly influence the apoptosis threshold of infected cells (Barzanti et al. 2001; Nakamura et al. 2003; Truong et al. 2003). Most anticancer drugs achieve their antitumoral effect by inducing apoptosis (Dixon et al. 1997; Johnstone et al. 2002), whereas any process that sets the apoptosis threshold higher may lead to multi-drug resistance (Morin 2003; Truong et al. 2003). In our experience with skin samples infiltrated by malignant MCs, there were no super-infections if cultures were maintained for 5 days, as corroborated by other authors (Nocka et al. 1990; Nakamura et al. 2003) and it seems that this culture period may be even extended (Li et al. 1991). In our opinion, a period of culture longer than 5-days may stress cultured cells, resulting in a shortened cellular lifetime, down expression of Ki67 and reduction of apoptotic rate. Ki67, a marker of cell proliferation in solid and haematological malignancies (Bennett et al. 1992; Gore et al. 1993), was detected in some neoplastic and non-neoplastic cells until 120 h of culture, indicating that the tissue was still viable and able to proliferate. A significant down expression of Ki67 recorded at 72, 96 and 120 h in treated cultures suggests that STI-571 has antiproliferative activity.

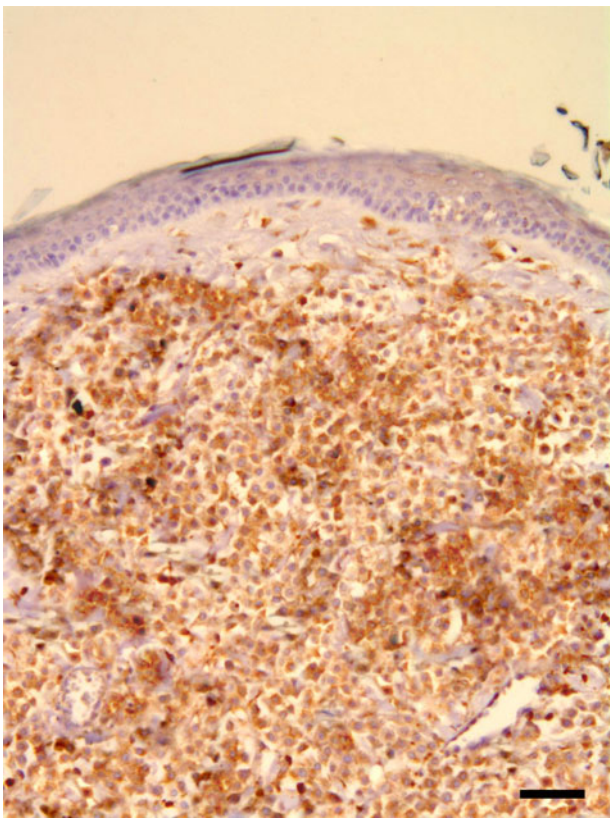

Fig. 4 Skin biopsy treated with STI-571. c-kit-positive malignant MCs at 48 h post-treatment (bar=25 μm)

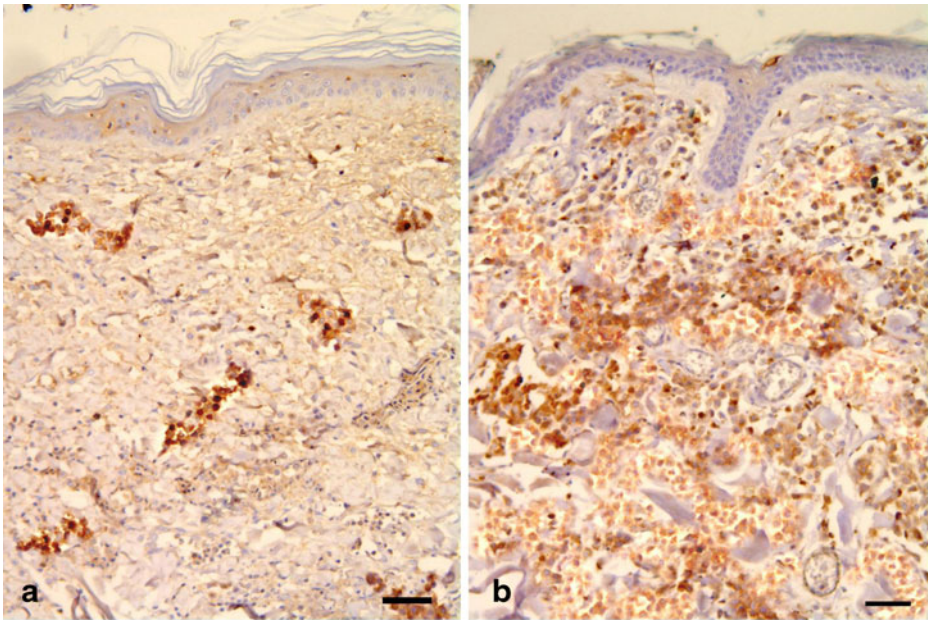

Fig. 5 **a** Skin biopsy treated with STI-571 at 96 h post-treatment. **b** untreated biopsy at the same time. Note the reducion in c-kit staining in A (bar=25 μm)

Spontaneous MCT are one of the most common malignant neoplasm in dogs, and often behave in an aggressive manner, metastasizing to local lymph nodes, liver, spleen, and bone marrow. The proto-oncogene c-kit is known to play a critical role in the development and function of MCs. Several studies of canine MCT have described mutations involving c-Kit, leading to a constitutively activated product and resulting in aberrant Kit protein localization (London et al. 1999; Downing et al. 2002; Zemke et al. 2002). Amplification of c-Kit may be a key factor in the malignant transformation of MCs, but it is currently unknown whether c-kit mutations and increased Kit expression are co-dependent or independent events. Kiupel et al. (2004) demonstrated a relationship between increased Kit expression and a more aggressive biologic behaviour of MCT. This relation may be explained by the functional role that Kit and its ligand SCF, play in MCT development. Kit and SCF have been shown to mediate numerous roles in MCT development, including proliferation, maturation, inhibition of apoptosis, adhesion, and migration (Nocka et al. 1990; Tsai et al. 1991; Yee et al. 1994). The exact mechanism by which increased Kit expression causes the malignant transformation of MCs is unknown. It has been suggested that the cytoplasmic isoform of Kit may be activated by soluble SCF or may contain constitutively activated mutations, thereby leading to the inhibition of apoptosis of MCs (Reguera et al. 2000).

Inhibitors of tyrosine kinases are promising anticancer agents that often induce apoptosis in neoplastic cells (Tsai et al. 1991). Imatinib mesylate is a tyrosine kinase inhibitor that has shown promising results in the treatment of CML and GIST (Nocka et al. 1989; Michels et al. 2002). A recent study has demonstrated that Imatinib mesylate has clinical activity against MCT in dogs. Response could not be predicted based on presence or absence of a mutation in exon 11 of c-Kit but all five dogs with a demonstrable c-kit mutation in exon 11 responded to the drug (Isotani et al. 2008). Mastinib, another potent and selective inhibitor of Kit, has shown to be safe and effective at delaying tumor progression in dogs presenting with recurrent or unresectable grade II or III non-metastatic MCT (Hahn et al. 2008).

According to our findings, STI-571 inhibits growth and induces apoptosis in canine MCT by a caspase-dependent way and indirectly decreases Bcl-2. Caspases are a family of aspartate-specific cysteine proteases activated by various apoptogenic signals that induce morphological features of apoptosis (Strasser et al. 1995; Thornberry and Lazebnik 1998). In the classic model, caspases are divided into initiator caspases (such as caspases-8 and 9) and executioner

Table 1 Results of two-way analysis of variance for the parameters measured in biopsy, before and after the STI-571 treatment

	Bcl-2	MCs	Caspase-3	c-Kit	Ki76	Tunel
1 (STI-571 treated)	$P<0.0005$	$P<0.0005$	$P<0.00001$	$P<0.0005$	$P<0.0005$	$P<0.00005$
2 (Time)	n.s.	$P<0.05$	$P<0.005$	$P<0.05$	$P<0.05$	$P<0.00005$
1×2 (STI-571 treated×Time)	$P<0.05$	$P<0.05$	$P<0.005$	$P<0.05$	$P<0.05$	$P<0.01$

caspases (such as caspases-3, 6 and 7), according to their function and their sequence of activation. Caspase-3 seems to play a central role in chemotherapy-induced apoptosis, by converting procaspase-3 into caspase-3.

This study demonstrated that all cultured biopsies have TUNEL-positive neoplastic MCs, suggesting that a higher apoptosis rate is present in both untreated and treated MCs, likely induced by the in vitro condition. Nevertheless, more apoptotic cells are present in treated than in untreated samples. Additionally, it was not surprising that in treated samples, Caspase-3-positive cells were more numerous than TUNEL-positive cells. In fact, cleaved caspase-3 positivity indicates ongoing apoptosis by active caspase, whereas the TUNEL method detects DNA fragmentation which evolves over a finite time period (Gown and Willingham 2002). In our opinion, the scattered caspase-positive cells in the untreated samples reflect the small effective number of apoptotic cells, suggesting that not all apoptotic-TUNEL-positive cells show a caspase-3 dependent way.

The severe loss of Bcl-2 protein expression observed in the treated neoplastic MCs confirmed that STI-571 induced apoptosis through a mechanism involving down-regulation of anti-apoptotic Bcl-2 family (Rahmani et al. 2005; Yu et al. 2005). Similarly, a dramatic decrease of Kit positive MCs in treated samples could be related to the STI-57-induced selective apoptosis by its capacity to trap Kit in an inactive configuration (Van Etten 2004). Interestingly, all neoplastic MCs surviving 120 h PT showed a type III pattern of Kit expression, suggesting the presence of acquired resistance to STI-571. As previously mentioned, STI-571 works in part by blocking the ATP binding pocket of kinases, such as Bcr/Abl fusion protein (in human patients with CML) or Kit. Different studies report an acquired cellular resistance to STI-571, most likely due to Bcr/Abl gene amplification or to mutations in the ATP-binding domain, thereby leading to the development of a second generation of Bcr/Abl kinase inhibitors, characterized by an enhanced activity (Talpaz et al. 2006).

In conclusion, in this study we demonstrated caspase-3 dependent apoptotic cell death in grade II MCT with mutations in c-Kit exon 11, induced by STI-571, through Kit inactivation and Bcl-2 downregulation. Grade II MCT are characterized by the coexistence of biologically different population of neoplastic MCs. Our results demonstrated that cellular sensibility to STI-571 is different, as surviving cells showed a common pattern of Kit distribution, scored as type III. We hypothesised that the STI-571-resistent cells more likely developed kinase domain mutants, thus preventing drug binding and eventually leading to recurrence in-vivo. Finally, this is the first report of a new system that mimics the treatment of real patients, thereby being helpful in the development of novel therapeutic strategies and in the prediction of drug sensitivity.

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