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# Imatinib interferes with survival of multi drug resistant Kaposi's sarcoma cells

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**Abstract Multi drug resistance (MDR) is defined as the ability of tumor cells to become resistant to unrelated drugs. Tyrosine kinase inhibitor imatinib has been demonstrated to be effective in the treatment of certain tumors. In particular, imatinib inhibits Bcr-Abl kinase activity, c-kit and the phosphorylation of platelet-derived growth factor (PDGF) receptors. In this work, we show that imatinib inhibits PDGF phosphorylation not only in *wt* Kaposi sarcoma (KS) but also in multi drug resistant KS cells. This was associated with an increased apoptosis in *wt* cells and an increased autophagy in MDR-KS cells. These data add new insights to the possible use of imatinib in the overcoming of MDR in KS cells.**


*Keywords:* Imatinib; Kaposi's sarcoma; Drug resistance; Apoptosis; Autophagy

## 1. Introduction

Receptor tyrosine kinases have been proposed as potential targets for antitumor therapy. Imatinib mesylate (also known as STI571 or Gleevec, and hereafter called imatinib) belongs to the group of new drugs classified as signal transduction inhibitors and has been approved as an effective treatment for Chronic Myeloid Leukaemia [1]. Imatinib inhibits Bcr-Abl kinase activity, causing apoptosis in Philadelphia$^+$ cells and inducing cytogenetic remissions in the majority of CML patients [1]. Additional tyrosine kinases are inhibited by imatinib: c-kit, the receptor for kit ligand (KL) and the two structurally similar platelet-derived growth factor receptors (PDGFRs), PDGFR-α and PDGFR-β [1,2]. Results of recent clinical studies have shown that imatinib therapy is well tolerated and leads to remission in patients with c-kit-positive gastrointestinal stromal tumors (GIST) that contain gain-of-function mutations in c-kit [3]. Imatinib has also been reported to inhibit the growth of several tumors, including Kaposi sarcoma (KS), all of which may express the PDGF/PDGFR or KL/c-kit autocrine growth loops [4–11]. Kaposi's sarcoma is a multi-focal angioproliferative disease that occurs in HIV-infected patients and is a leading cause of mortality and morbidity in the acquired immune deficiency syndrome (AIDS) [12]. A number of cytokines and growth factors have been implicated in KS progression [13,4]. In particular, activation of PDGF and c-kit receptors has been proposed to play a role in mediating the growth of AIDS-related KS [14]. Furthermore, although a reduced incidence and regression of KS have been reported in AIDS patients treated with antiretroviral therapies or with chemotherapy (e.g. by Doxorubicin), the drug toxicity and the appearance of multi drug resistance (MDR) represent the main cause of therapeutic failure. Here, we show that imatinib mainly induces apoptosis in KS cells and, more interestingly, autophagy in MDR-KS cells.

## 2. Materials and methods

### 2.1. Cells culture and treatments

The wild type Kaposi's sarcoma cell line (*wt*-SLK) and doxorubicin (DOX) resistant MDR$^+$ cells (SLK–DOX) were kindly provided by Dr. BM Lucia and Prof. Roberto Cauda (Catholic University, Rome, Italy). The cells were grown in RPMI-1640 supplemented with 10% FCS, 2 mM glutamine and antibiotics. SLK–DOX cells were selected from the original *wt*-SLK cell line by exposure to 50 ng/ml of the drug. Drug resistance was maintained by adding relevant concentrations of the drug every 4 weeks. Cells were cultured in drug-free medium for at least ten days before experimental procedures. Degrees of resistance were assessed in terms of MTT assay [15] and the P-gp and/or MRP function [16]. Cells were treated with imatinib (Novartis) at different concentrations (15, 25 and 35 μM) in the growth medium at 37 °C in a 5% $CO_2$ atmosphere for 48 h.

### 2.2. Cell growth

Cell proliferation was analyzed by performing growth curves both in *wt*-SLK and SLK–DOX cells. Cell number was determined by counting cells daily using the trypan blue (GIBCO, Loughbolough, UK) exclusion test.

### 2.3. Analytical cytology

For static and flow cytometry analyses, control and treated cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min at room temperature. After washing in the same

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*Abbreviations:* KS, Kaposi sarcoma; MDR, multi drug resistance; DOX, Doxorubicin; PDGFRs platelet-derived growth factor receptors; KL, kit ligand

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buffer, cells were permeabilized with 0.5 Triton X-100 (Sigma Chemical Co., St. Lois, MO, USA) in PBS for 5 min. For PDGF-β receptor (phosphorylated and non-phosphorylated) monoclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) directed against these antigens were used. For Bcl-2 and Beclin-1 (Santa Cruz Biotechnology) polyclonal antibody directed against these antigens were used. After 30 min at 37 °C, cells were washed and then incubated with an anti-mouse fluorescein-linked or anti-rabbit fluorescein-linked whole antibodies (Sigma). For apoptosis detection the nuclei were stained with Hoechst 33258 (Sigma) at 37 °C for 15 min. For a qualitative analysis all samples were mounted on glass cover-slips with glycerol–PBS (2:1) and analyzed by intensified video microscopy (IVM) with a Nikon Microphot fluorescence microscope equipped with a Zeiss CCD camera. Regarding flow cytometry analyses, all the samples were recorded with a FACScan flow cytometer (Becton–Dickinson, Mountain View, CA, USA) equipped with a 488 nm argon laser. A least 20000 events have been acquired. The median values of fluorescence intensity histograms were used to provide a semi-quantitative analysis.

### 2.4. Acid compartment detection

Autophagic vacuoles were labelled with the autofluorescent drug, monodansylcadaverine (MDC, Sigma), by incubating cells with 0.05 mM MDC in phosphate-buffered saline (PBS) at 37 °C for 10 min. Acid compartments were also labelled by incubating the cells with 1 μM LysoTracker (LTR, Molecular Probes, Eugene, OR, USA) in the culture medium at 37 °C for 15 min. Cells were then fixed with 4% paraformaldehyde (w/v in PBS) for 1 h at room temperature (25 °C). Cells were analyzed by IVM.

Regarding flow cytometry analyses, all the samples were evaluated with a FACScan flow cytometer (Becton–Dickinson) equipped with a 488 nm argon laser. At least 20000 events have been acquired. The median values of fluorescence intensity histograms were used to provide a semi-quantitative analysis.

### 2.5. Morphometric analyses

Quantitative evaluation of apoptotic cells was performed counting at least 300 cells at high magnification (500×) at the fluorescence microscope after Hoechst labeling.

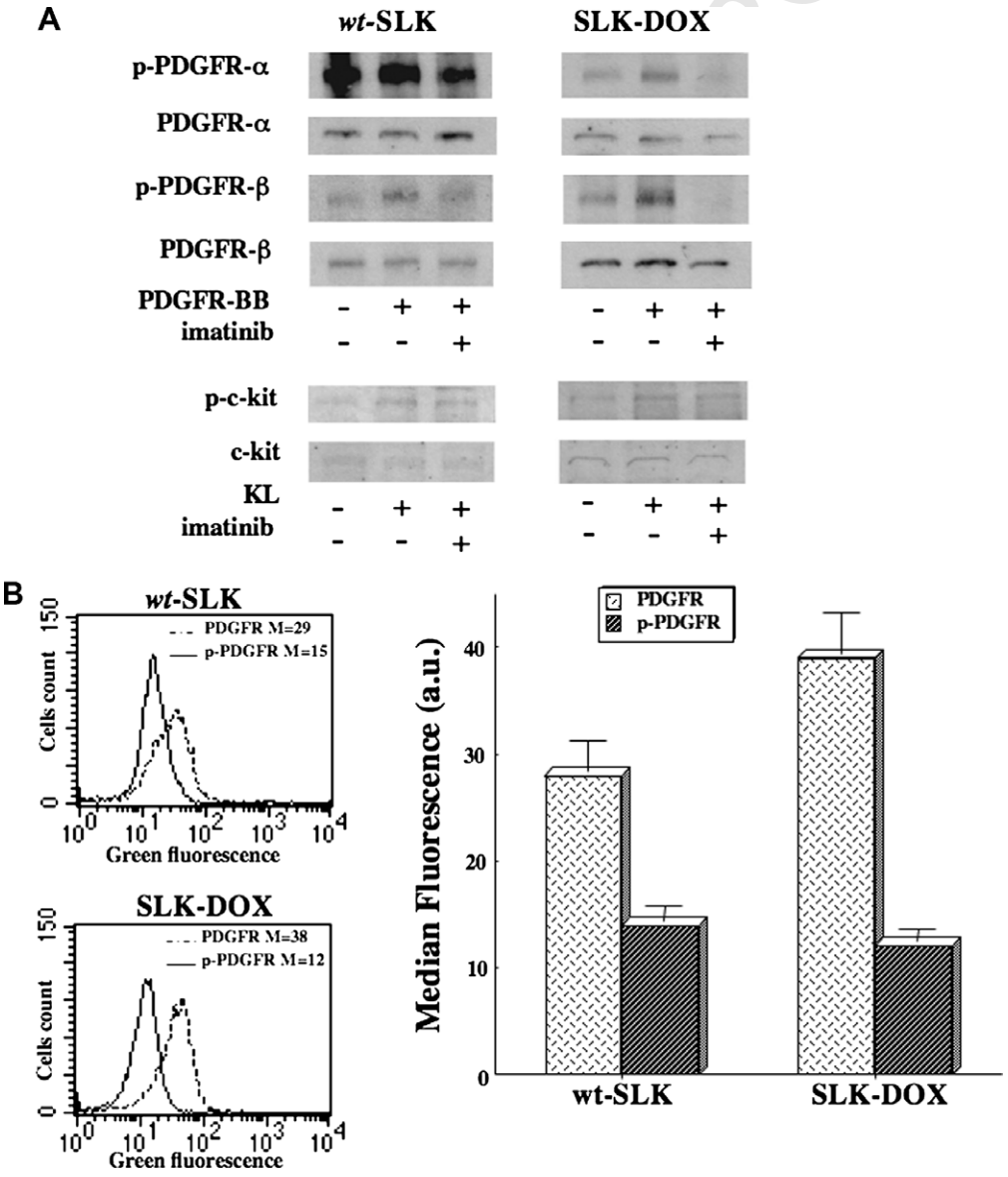


Fig. 1. Quantitative analysis of non-phosphorylated and phosphorylated PDGF receptors (α and β) and c-kit in both *wt* and resistant SLK cells by Western blotting (A). Flow cytometry evaluation of phosphorylated and non-phosphorylated PDGF receptors is shown in (B) as representative results (left panels) or as mean histograms (right panel, mean values ± S.D. from four independent experiments). Student's *t*-test to correlate samples was used.

### 2.6. Caspase activity

Activation state of the caspase 8, 9 and 3 was evaluated by using the CaspGLOW fluorescein active caspase staining Kit (MBL, Woburn, MA, USA). This kit provides a sensitive means for detecting activated caspases in living cells. The assay utilizes specific caspases inhibitors (IETD-FMK for caspase 8, LEHD-FMK for caspase 9 and DEVD-FMK for caspase 3) conjugated to FITC as the fluorescent marker. These inhibitors are cell permeant, non-toxic and irreversibly bind to caspase active form. The FITC label allows detection of activated caspases in apoptotic cells directly by flow cytometry. Cells were incubated with FITC-IETD-FMK, FITC-LEHD-FMK or FITC-DEVD-FMK for 1 h at 37 °C following manufacturer instruction. After this time samples were washed three times and immediately analyzed on a cytometer by using FL-1 channel. Two additional experimental controls were also considered: (i) samples prepared by pre-treating cells with specific inhibitors of caspase 8, 9 or 3 and (ii) unlabelled cells (negative control).

### 2.7. Western blotting

To study the effect on the phosphorylation of PDGF-α and PDGF-β receptors, subconfluent cells shifted to 0.1% FBS overnight, were cultured with and without 15 μmol/l imatinib for 4 h followed by an additional 10 min of incubation in the absence or in the presence of 10 ng/ml human recombinant PDGF-BB (Roche, Welwyn Garden City, UK). At the end of incubation, cells were washed with PBS, harvested and cells pellet was lysed in the ice-cold radioimmunoprecipitation assay (RIPA) buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM

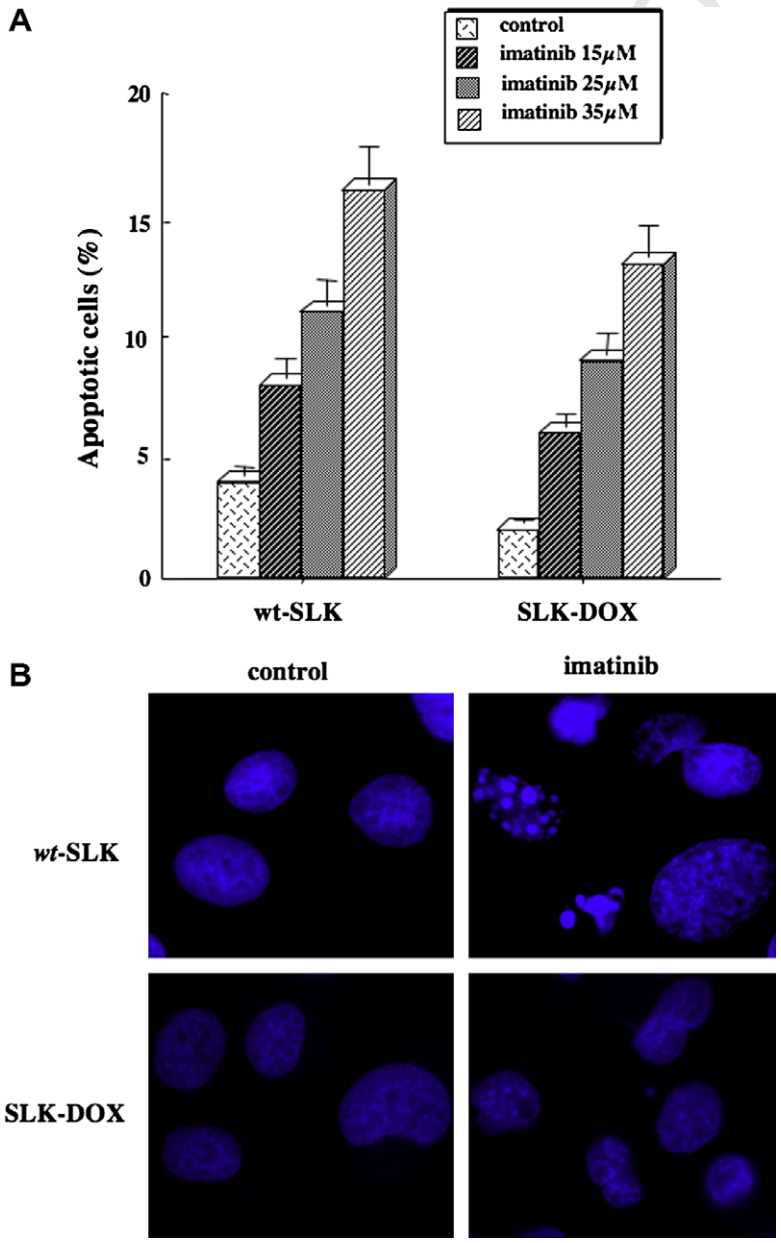


Fig. 2. (A) Morphometric analysis indicating the percentage of apoptotic cells in *wt* and resistant SLK cells exposed to different concentrations of imatinib for 48 h. Mean values from four independent experiments ± S.D. are shown. Note as percentage of apoptotic cells increases with increasing of imatinib concentration. (B) Fluorescence microscopy of *wt* and resistant SLK stained with Hoechst 33258. Note that the chromatin condensation occurred in both cell lines 48 h after imatinib exposure (right panels).

EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 200 mM PMSF, 1 mg/ml leupeptin, 1 mg/ml aprotinin plus 1% Triton X-100), followed by centrifugation at 14000 rpm at 4 °C for 20 min. Protein concentration was determined using the Bradford protein assay method. Equal amounts (80 μg) of sample protein were loaded onto 7% SDS–polyacrylamide gels and then electrotransferred onto nitrocellulose membranes which were incubated with 1:300 dilution of the following primary antibodies: affinity-purified polyclonal rabbit anti-phospho-PDGFR-α (anti-p-PDGFR-α, Sigma), anti-phospho-PDGFR-β (anti-p-PDGFR-β, Sigma) and monoclonal mouse anti-actin (Santa Cruz Biotechnology). Membranes were then incubated with the horseradish peroxidase-conjugated secondary antibodies (1:10000 dilution; Santa Cruz Biotechnology). Detection was performed using enhanced chemiluminescence (ECL, Amersham Life Science, Arlington Heights, IL). Secondary antibodies alone served as negative controls. Protein bands were quantified by densitometric analysis using a densitometry computer software (Kodak Digital Science, Rochester).

### 2.8. Statistical analyses

Cytofluorimetric results were statistically analyzed by using the parametric Kolmogorov–Smirnov test using Cell Quest Software. Morphometric data (reported as mean ± standard error, S.E. from at least four separate experiments) were analyzed by using Student *t*-test. Only $P < 0.05$ was considered as significant.

## 3. Results and discussion

### 3.1. PDGFR expression

We first examined non-phosphorylated and phosphorylated PDGFR-β, PDGFR-α and c-kit expression in both *wt* and resistant SLK cells by Western blotting and flow cytometry. As shown in Fig. 1A, both *wt*-SLK and SLK–DOX cells expressed the PDGF receptors, whilst c-kit was barely detectable. Ligand-induced phosphorylation of PDGFR-β, PDGFR-α and c-kit was inhibited to background levels in cells treated with imatinib without affecting the expression levels of the receptors. A high expression of β-subtype non-phosphorylated receptor as detected by flow cytometry is also shown in Fig. 1B. Since it was suggested that inhibition of PDGFR phosphorylation is associated with apoptosis induction [17],

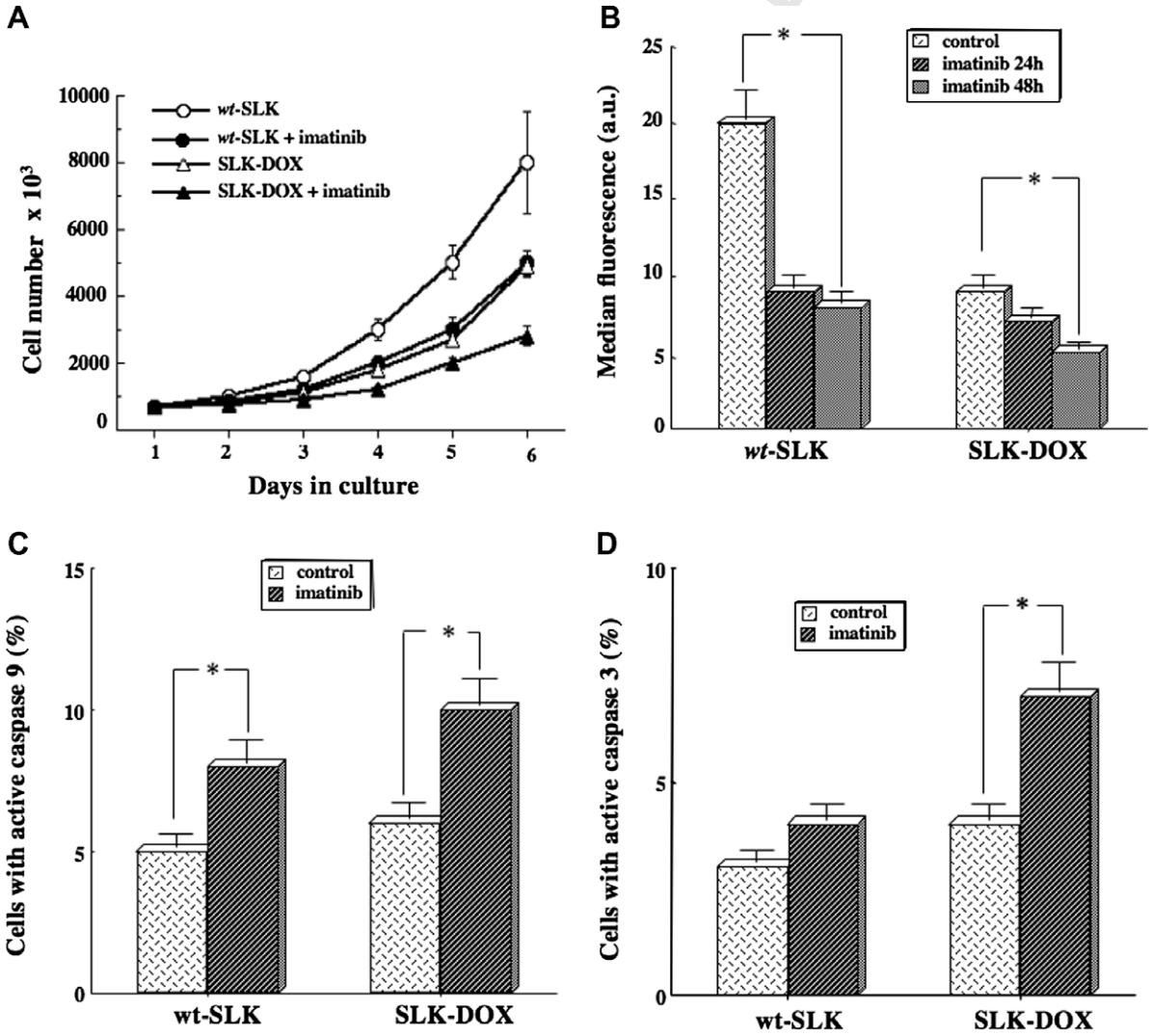


Fig. 3. (A) Cell proliferation is inhibited by treatment with imatinib 15 μM in both *wt* and SLK–DOX cells. (B) Quantitative analysis of Bcl-2 protein by flow cytometry. Note the higher expression of this molecule in *wt* cells with respect to MDR+ cells. Also note that in both cell lines the expression of this protein was significantly decreased 24 and 48 h after imatinib exposure. Data are reported as mean values ± S.D. from four independent experiments. Student's *t*-test to correlate samples was used ($^*P < 0.01$). (C, D) Activation state of caspase 9 (C) and 3 (D) evaluated by flow cytometry in both *wt* and SLK–DOX cells. Note that, especially in SLK–DOX cells, these caspases are activated significantly by imatinib. Data are reported as mean values ± S.D. from four independent experiments. Student's *t*-test to correlate samples was used ($^*P < 0.01$).

experiments were carried out in order to verify if imatinib-induced apoptosis and the mechanism involved.

### 3.2. Apoptosis and cell growth evaluation

In order to verify if imatinib could induce apoptosis, we exposed both SLK cell lines to different concentrations of imatinib (15, 25, 35 μM for 48 h). As shown in Fig. 2A, imatinib was able to induce apoptosis in a dose dependent manner in both cell lines. In Fig. 2B the typical sign of apoptosis, i.e. chromatin condensation, is shown. Moreover, considering that the plasma steady state levels of imatinib observed in treated patients correspond to values between 3 and 10 μM [18], we focused our attention on the effects induced by imatinib at 15 μM on both *wt* and resistant SLK cells. In particular, as reported

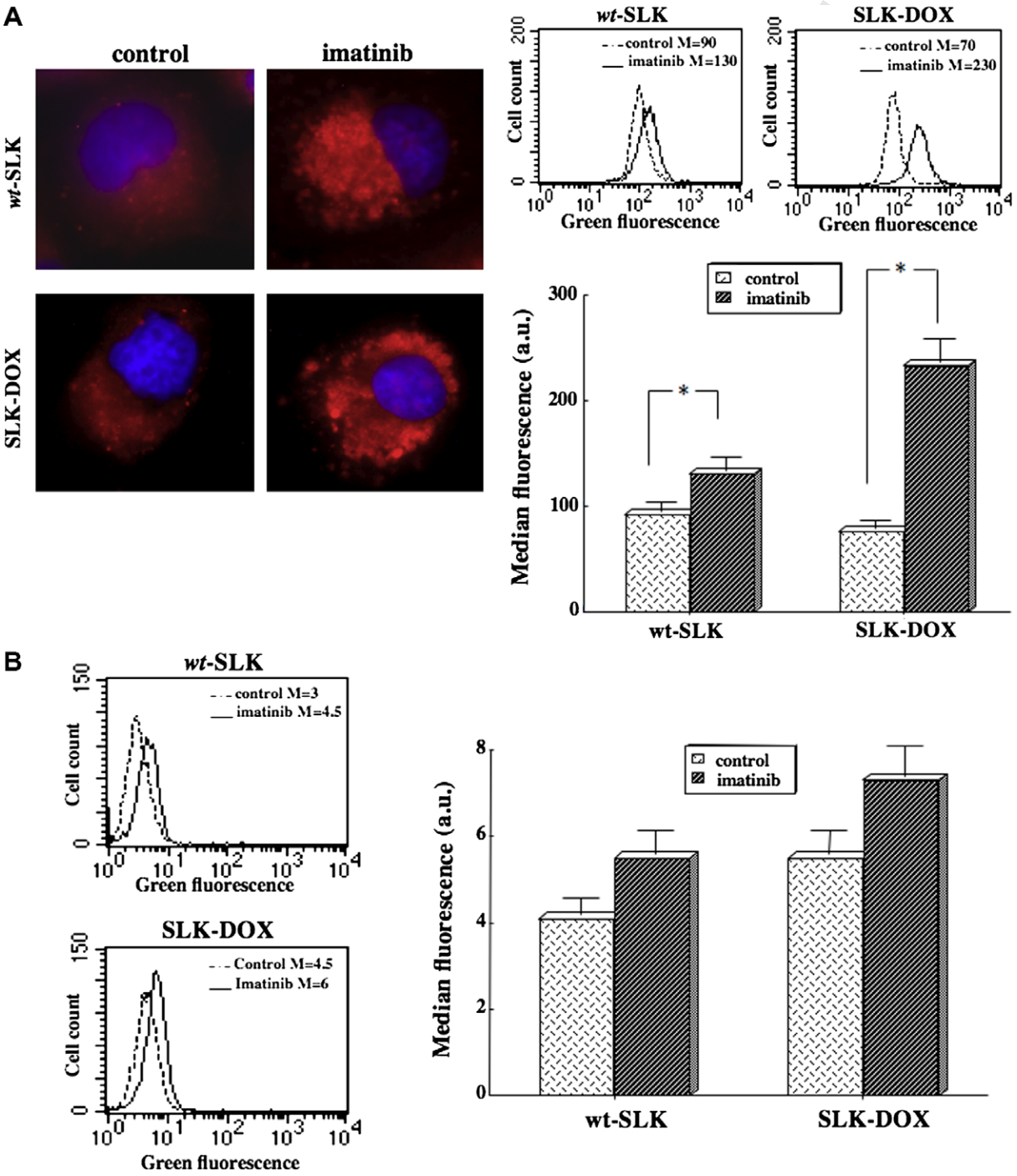


Fig. 4. (A) IVM analysis showing the presence of diffuse lysosomal vacuoles in cells exposed to imatinib (left). Quantitative analysis of lysosomal vacuoles obtained by flow cytometry from four independent experiments. Note the significantly increased lysosome acidification after imatinib treatment in SLK–DOX cells (right). (B) Quantitative evaluation of Beclin1 expression in a representative experiment (left). Mean values histograms (right) obtained from four independent experiments ± S.D. In ordinate, median fluorescence intensity is reported. Student's *t*-test to correlate samples was used ($^{*}P < 0.01$).

in Fig. 3A, a significantly decreased cell grow was detected after 15 μM imatinib exposure in both *wt* and SLK–DOX cells. Hence, imatinib was capable of impairing cell growth and to induce apoptosis in both *wt* and MDR-KS cells.

### 3.3. Bcl-2 expression

We then analyzed the expression of Bcl-2, an integral membrane protein mainly located on the outer membrane of mitochondria. Its overexpression prevents cells from undergoing apoptosis in response to a variety of stimuli [19]. In our experiments, a quantification of this protein was carried out in both *wt* and resistant SLK cells. As shown in Fig. 3B, the expression of this protein decreased significantly 24 and 48 h after imatinib treatment in both cell lines.

### 3.4. Caspase activity

Apoptosis occurs through two main pathways. The first, referred to as the extrinsic or cytoplasmic pathway, is triggered through the death receptors. The second is the intrinsic or mitochondrial pathway that, when stimulated, leads to the release of cytochrome *c* from the mitochondria and the activation of death signals. Both pathways involve the activation of a cascade of proteases called caspases that cleave regulatory and structural molecules, culminating in the death of the cell. Considering that intrinsic and extrinsic pathways converge to caspase 3 by caspase 8 (in the extrinsic pathway) and by caspase 9 (in the intrinsic pathway) we evaluated the activation state of these proteases in both *wt* and SLK–DOX cells. Data shown in Fig. 3 indicate that imatinib was able to induce apoptosis by activating caspase 9 (Fig. 3C) and 3 (Fig. 3D). Interestingly, imatinib-induced caspase activation was significantly higher in SLK–DOX cells with respect to *wt* cells. No differences were detected in the activation state of caspase 8 (data not shown). This may suggest that mitochondrial pathway could play a key role in imatinib-induced cell death.

### 3.5. Imatinib and lysosomal function

Autophagy is an archetypical cellular degradation mechanism in which an increase of lysosomal compartment and the formation of autophagic vacuoles can be detected [20]. It has recently been suggested that imatinib is capable of inducing autophagy and that this might represent an additional mechanism leading to growth arrest, apoptosis and tumor regression [21]. To determine whether imatinib modulated endolysosomal compartment in KS cells, a specific lysosomal marker, the lysotracker, was used. This probe allowed us to investigate the alteration of lysosomal function, i.e. the acidification of lysosomes and or their trafficking in cells. Analyses conducted by fluorescence microscopy revealed the presence of diffuse lysosomal vesicles in cells exposed to imatinib with respect to untreated cells (Fig. 4A). Quantitative evaluations, obtained by flow cytometry analyses, emphasized an increased lysosome acidification after imatinib treatment. Importantly, this increase was much more evident in MDR-KS cells, i.e. in SLK–DOX cells (Fig. 4A). Similar results were obtained by using monodansylcadaverine, an autophagosomal marker (data not shown). Finally, these results were confirmed by the analysis of Beclin-1/ATG6 [22].

The results obtained emphasized a link between apoptosis and autophagy induced by imatinib. Autophagy induction could be considered as an additional mechanism of imatinib to induce growth arrest and promote cell death in sarcoma cells. Moreover, considering that resistance to chemotherapeutic agents predominantly occurs through defects in the apoptotic signaling pathway [23], the identification of agents, such imatinib, that can stimulate autophagy may become of interest in the clinical management of Kaposi's sarcoma. In fact, the possibility that the inhibition of PDGFR phosphorylation by imatinib could trigger a series of cellular pathways leading to cell demise by apoposis (in *wt* cells) or inducing autophagy (in $MDR^+$ cells) can also open new therapeutic strategies, e.g. in terms of combinatory drug therapies.

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