Dasatinib Stimulates Its Own Mechanism of Resistance by Activating a CRTC3/MITF/Bcl-2 Pathway in Melanoma with Mutant or Amplified c-Kit

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ABSTRACT

Amplification or activating mutations of c-Kit are a frequent oncogenic alteration, which occurs commonly in acral and mucosal melanoma. Among c-Kit inhibitors, dasatinib is the most active due to its ability to bind both active and inactive conformations of the receptor. However, its use as a single agent in melanoma showed limited clinical benefit. We first found that sensitivity to dasatinib is restricted to melanoma cell lines harboring c-Kit alteration but, unexpectedly, we observed lower effect at higher concentrations that can readily be found in patient blood. We then investigated relevant pathway alterations and found complete inhibition of MAPK and PI3K/AKT pathways but an increase in MITF and its downstream target Bcl-2 through CRTC3 pathway, which turn on the CREB regulated transcription of MITF. More importantly, dasatinib upregulates MITF and Bcl-2 through SIK2 inhibition revealed by CRTC3 reduced phosphorylation, CREB transcription activation of MITF, MITF transcription activation of Bcl-2 as well as

pigmentation. Furthermore, overexpression of MITF renders melanoma cells resistant to all dasatinib concentrations. Selective Bcl-2 inhibition by ABT-199 or Bcl-2 knockout restores the sensitivity of melanoma cells to dasatinib, validating the involvement of MITF and Bcl-2 axis in the resistance of melanoma to dasatinib. In conclusion, we showed for the first time that dasatinib in melanoma stimulates its proper mechanism of resistance, independently of MAPK and PI3K/AKT pathways reactivation commonly associated to secondary c-Kit mutations, but through CRTC3/MITF/Bcl-2 pathway activation at clinically relevant doses which may explain the weak clinical benefit of dasatinib in patients with melanoma.

Implications: Dasatinib stimulates its proper mechanism of resistance through CRTC3/MITF/Bcl-2 pathway, which may explain its modest clinical efficiency in patients with melanoma.

Introduction

Melanoma is a malignant tumor, which arises from the malignant transformation of melanocytes. It is the least common but the most aggressive skin cancer, accounting for the vast majority of skin cancer death (1, 2). Melanoma incidence has increased over the past 50 years (3). MAPK pathway is continuously activated in about 75% of all cutaneous melanomas through activating mutations in BRAF (v-raf murine sarcoma viral oncogene homolog B1) and NRAS [neuroblastoma RAS viral (v-ras) oncogene homolog]. In physiologic conditions, this pathway as well as PI3K/AKT pathway are regulated by receptor tyrosine kinases such as *KIT* (c-Kit; ref. 4). c-Kit is a proto-oncogene located in a region on the long arm of chromosome 4 (4q11–4q13), which also encodes for the SCF receptor (CD117) and is found mutated in all melanoma subtypes but most frequently (20%–40%) in melanoma arising on mucosal membranes, acral skin, and skin with

chronic sun-induced damage (CSD; refs. 5, 6). Ligand-independent activation of c-Kit can be caused by gain-of-function mutations as well as gene amplification, which have been reported in several tumors including melanoma (6). The identification of c-Kit mutations in cancer led to the development of a number of small molecule inhibitors that are now under intensive preclinical and clinical investigations. Among these is dasatinib an orally available small-molecule multi-kinase inhibitor (7), which showed promising results in preclinical studies in various solid tumors, including prostate cancer (8), breast cancer (9), and melanoma (10). Indeed, studies using melanoma cell lines reported significant inhibitory effect of dasatinib on cell proliferation, survival, migration, invasion, and proposed its combination with standard chemotherapy. In parallel, clinical trials using dasatinib in patients with melanoma showed efficacy in a small subset of unselected patients with melanoma (11). Although at the beginning, preclinical studies suggested a higher potential for dasatinib than for other c-Kit inhibitors (12), these observations did not translate clinically. The positive expectations were based on the fact that dasatinib is thought to bind to both active and inactive conformations of c-Kit as opposed to imatinib (FDA approved drug in melanoma), which only binds to the inactive structure of c-Kit. This difference was believed to make dasatinib more potent and effective than imatinib (13, 14). Therefore, dasatinib (Sprycel, BMS) was tested in a phase II trial including patients with solar, mucosal, and acral melanoma (NCT00700882). Again dasatinib reveals modest clinical efficacy due to not fully understood mechanisms of resistance and ask the question why c-Kit mutant melanoma patients do not respond to the treatment (15).

Microphthalmia-associated transcription factor (MITF) is a basic helix–loop–helix master transcription factor regulating cell growth and differentiation in the melanocyte. The transcriptional activity of MITF protein is modulated by protein kinase cascades that are induced

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by the stem cell factor and its receptor kinase, c-Kit (16). In addition, signaling downstream of melanocyte-stimulating hormone/melano-cortin 1 receptor (MSH/MC1R) triggers cAMP production, leads to protein kinase A (PKA)-mediated salt inducible kinases (SIK) family member phosphorylation, which block SIKs ability to access and phosphorylate their substrate CREB-regulated transcription coactivator 3 (*CRTC3*). When dephosphorylated, CRTC3 translocates into the nucleus, activates cAMP-response element binding (*CREB*), which consequently stimulates MITF expression. Once activated, MITF-target genes regulate melanocyte pigmentation (by mechanisms that include the induction of tyrosinase and *TYRP-1*). Transcriptional stimulation of several melanogenic genes is mainly mediated by cAMP-dependent activation of MITF (17–19). Reducing CRTC phosphorylation via small molecule SIK inhibitors showed efficiency to stimulate MITF expression and pigmentation even in the absence of increased cellular cAMP levels (20, 21). Despite that bosutinib and dasatinib were developed for other purposes, they were described as SIK inhibitors, prompting CRTC3 reduced phosphorylation and consequently favoring MITF expression and pigmentation (22, 23). In addition, MITF exhibit pro-survival function in melanoma through regulation of the expression of several anti-apoptotic genes such as B-cell lymphoma 2 (*BCL2*, Bcl-2), Bcl-2-related protein A1 (*BCL2A1*), and ML-IAP/livin (*BIRC7*; ref. 24). On the basis of biopsies, derived from melanoma tissue patients or xenograft, MITF expression was found upregulated in response to MAPK inhibition. MITF was described involved in the resistance to the MAPK pathway inhibitors through various mechanisms, such as enhanced survival signaling and altered metabolism (25). One of the main targets of MITF involved in melanoma survival and drug resistance is the anti-apoptotic Bcl-2. Coherently, it was shown that in melanoma, MITF proteins occupy the endogenous Bcl-2 promoter and consequently an increase or decrease in MITF expression modulates Bcl-2 expression. Unfortunately, this MITF/Bcl-2 axis was described as an effective mechanism of resistance to treatment in melanoma (26, 27). Increase of Bcl-2 levels alternate the apoptotic response to cytotoxic chemotherapy and its dysregulation affect melanoma cell survival and drug resistance (28). In BRAF and NRAS mutant melanoma, Bcl-2 inhibition enhanced response to ^{V600}BRAF inhibitors and MEK inhibitor (26, 28).

In this work, we identified the CRTC3/MITF/Bcl-2 pathway as a main resistance mechanism to dasatinib activated early under relatively high doses that could be achieved in patient blood despite a complete inhibition of c-Kit, MAPK, and PI3K/AKT pathways. The activation of this pathway may explain the discrepancy between scientific expectations, *in vitro* promising results and the modest clinical response to dasatinib.

Materials and Methods

Inhibitors and effectors
The c-Kit/SRC inhibitor "dasatinib" was kindly provided by Bristol Myers Squibb. The Bcl-2 inhibitor "ABT-199" was from Selleck Chemicals. The cAMP inducer "forskolin" (FSK) was from Calbiochem.

Ethics
Human melanoma cell lines used in this study were all established in our Laboratory of Oncology and Experimental Surgery, Institut Jules Bordet, Belgium, the majority from skin and lymph node metastases. Of note all lines used in this study originate from patients in accordance with the Declaration of Helsinki and with Good Clinical Practice guidelines as defined by the International Conference on Harmoni-

zation. All patients provided written and signed informed consent before enrollment. M230 cell line was kindly provided to Lady Davis Institute, McGill University, Canada, by Dr A. Ribas (UCLA, Los Angeles, CA, USA). All cell lines in this study are continuous cell lines with more than 100 passages and were regularly checked for mycoplasma contamination using MycoAlert Mycoplasma Detection Kit (Lonza). The study was approved by the review board of the ethical committee of "Institut Jules Bordet," Brussels, Belgium. Cells authentication was evaluated as described previously (29). BRAF NRAS, KIT mutations were assessed with the next-generation DNA sequencing for 48 genes from cancer panel (TruSeq Amplicon - Cancer Panel), performed in the Pathology Department of Institut Jules Bordet.

Establishment of a cell line with acquired resistance to dasatinib (termed HBL-R)
To mimetic patient resistance to targeted therapy and to explore the long-term effect of dasatinib treatment, we established a c-Kit mutant melanoma cell line with acquired resistance to dasatinib by exposing parental cells (HBL) to gradually increasing concentrations of dasatinib (from 10^{-11} to 10^{-6} M) over a period of twelve weeks resulting in 500-fold increase of IC_{50} (**Fig. 2C** and **D**).

Cell culture conditions
Cells were grown in Ham's F10 medium supplemented with 5% heat-inactivated FCS, 5% heat-inactivated new-born calf serum, L-glutamine, penicillin, and streptomycin at standard concentrations (all from Gibco) at 37°C in a humidified 95% air and 5% CO_2 atmosphere. For routine maintenance, cells were propagated in flasks, harvested by trypsinization (0.05% trypsin-EDTA) (Gibco), and subcultured twice weekly.

Proliferation assay
Cell proliferation was assessed by crystal violet staining method. All cells were seeded in 96-well plates (8,000 cells/well) using complete Ham's F10 medium. One day after plating, the culture medium was replaced by a fresh one containing or not inhibitors/effectors depending on experimental conditions and cells were cultured for three additional days. Then, culture medium was removed and cells were gently rinsed with PBS, fixed with 1% glutaraldehyde for 15 minutes, and stained with 0.1% crystal violet (w/v in water) for 30 minutes. Cells were destained under running tap water and subsequently lysed with 0.2% (v/v in water) Triton X-100 for 90 minutes. The absorbance was measured at 570 nm using a Multiskan EX Microplate Photometer (Thermo Fisher Scientific). On each plate, blank wells containing medium alone were used to estimate background.

Apoptosis determination
Apoptotic cells were detected by Annexin-V/PE Apoptosis Detection Kit I (BD Pharmingen), according to the manufacturer's recommendations. Cells were seeded in 6-well plates (2×10^5 cells/well) in culture medium. One day after plating, the culture medium was replaced by a fresh one containing or not inhibitors/effectors and cells were further incubated for 2 days prior to assay and analyzed in a flow cytometer (FACS Calibur; Becton Dickinson).

Western blot analysis
Cells were plated in 160 cm^2 Petri dishes (3×10^6 cells/dish) in culture medium. One day after plating, the culture medium was replaced by a fresh one and further left for 2 days. Cells were exposed or not to effectors for 24 hours. Cells were lysed using a detergent cocktail (M-PER mammalian extraction buffer) supplemented

Figure 1.
Characterization of dasatinib sensitive melanoma cells. **A,** Effect of increasing concentrations of dasatinib (10^{-12}–10^{-4} M) on the proliferation of different melanoma cell lines, 3 days after treatment. Crystal violet staining. Data are presented as means $\pm$ SEM ($n = 3$ experiments). **B,** Western blot analysis of phosphorylated and unphosphorylated form of c-Kit, SRC, ERK, and AKT in the three group of melanoma cell lines with different sensitivity to dasatinib. The samples of each group were lysed at the same time and analyzed in the same order on separate gels to evaluate different proteins expression relative to β-actin.
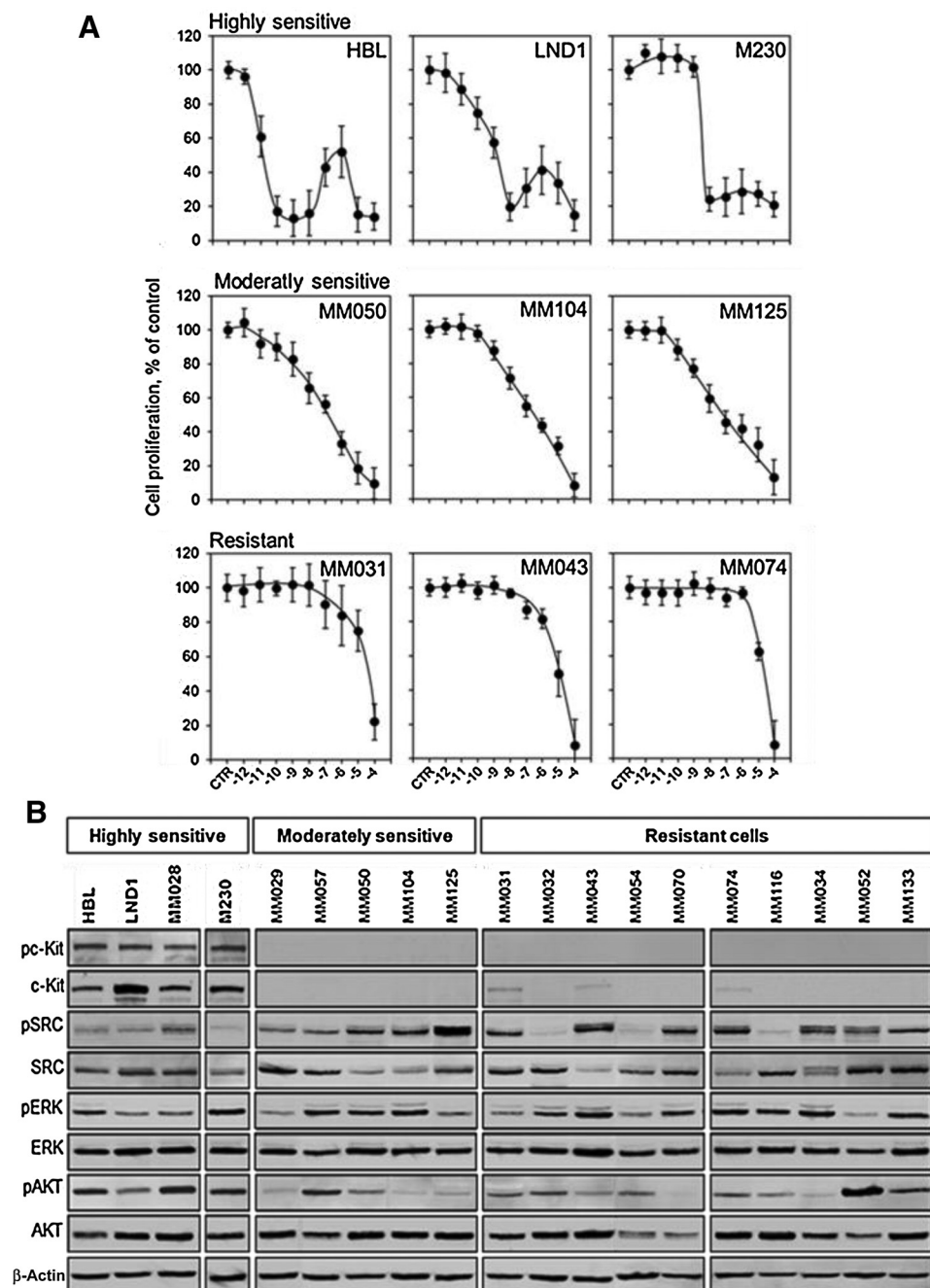

with protease inhibitors (Halt protease inhibitor cocktail) and phosphatase inhibitors (Halt phosphatase inhibitor cocktail; all from Pierce). Protein concentrations were determined by the BCA Protein Assay (Pierce BCA Protein Assay Kit; Thermo Fisher Scientific) using BSA as standard. immunodetections used antibodies raised against c-Kit (1/1,000), phospho-SRC (Tyr 416; 1/1,000), SRC (1/1,000), phospho-AKT (Ser 473; D9E, 1/500), AKT (1/2,000), Bcl-2 (1/1,000), MITF (1/1,000; all from Cell Signaling Technology), and phospho-c-Kit (Tyr 703; 1/200), phospho-ERK (Tyr 204; E-4, 1/1,000), ERK-2 (C-14, 1/2,000), SIK2 (1/100; from Santa Cruz Biotechnology), phospho-CRTC3 (Ser 370; 1/2,000; from Abcam), and β-actin (1/5,000; from Millipore). Peroxidase-

labeled anti-rabbit IgG antibody (1/5,000) or peroxidase-labeled anti-mouse IgG antibody (1/5,000; both from GE Healthcare Europe GmbH) were used as secondary reagents to detect corresponding primary antibodies. Bound peroxidase activity was revealed using the SuperSignal West Pico Chemiluminescent Substrate (Pierce). Relative protein expression (fold change) were calculated in reference to β-actin and then normalized by the control protein.

CRISPR/Cas9 Bcl-2 knockout (KO)

Cells were transfected with CRISPR/Cas9 plasmids (Bcl-2 CRISPR/Cas9 KO or control CRISPR/Cas9 and Bcl-2 HDR; Santa Cruz Biotechnologies) using UltraCruz transfection reagent according to

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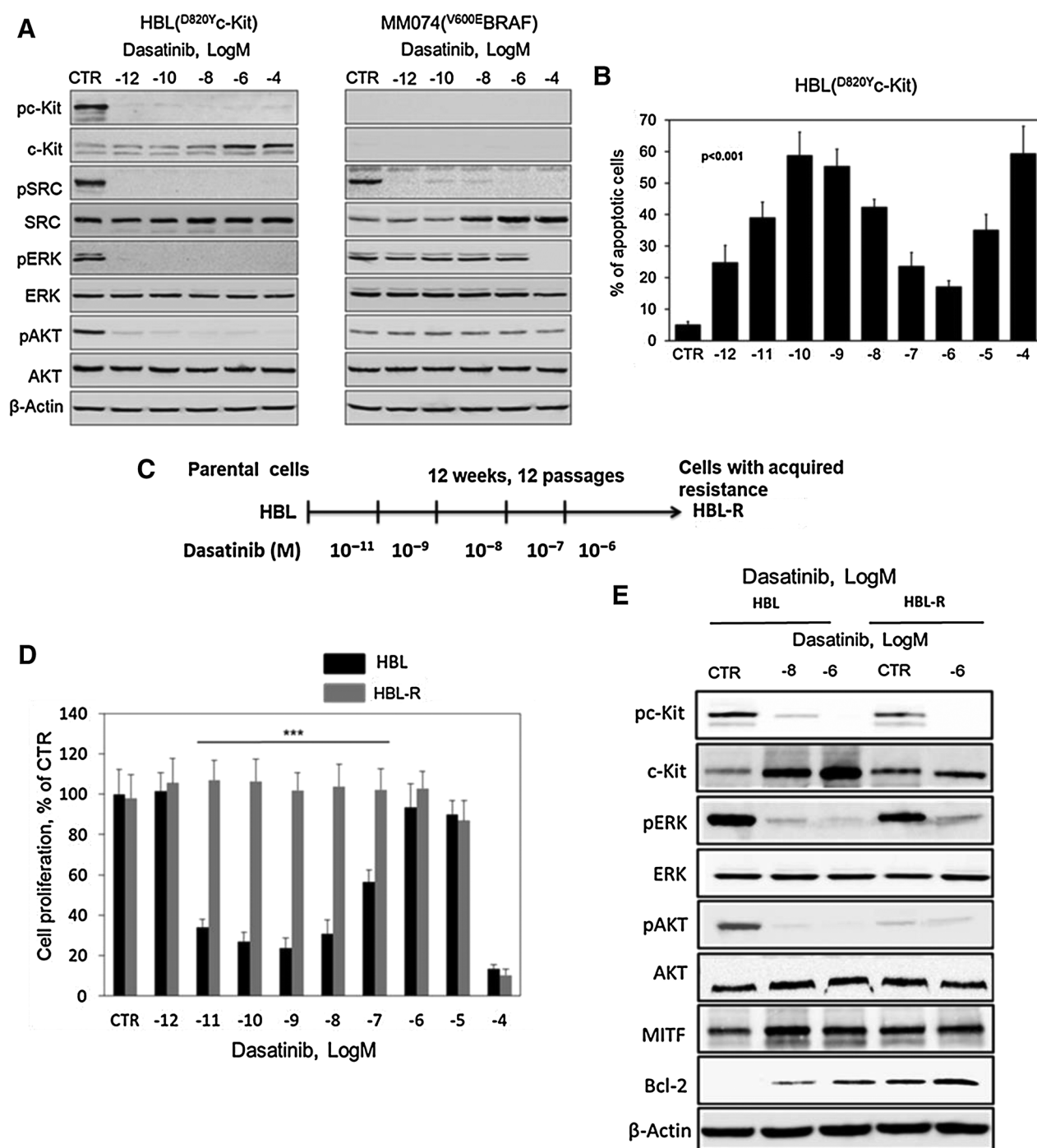
Figure 2.
Resistance to dasatinib is associated with MITF/Bcl-2 pathway activation. **A,** Western blot analysis of expression and phosphorylation of c-Kit, SRC, AKT, and ERK in HBL (highly sensitive to dasatinib) and MM074 (resistant to dasatinib) cells exposed to doubling concentrations of dasatinib (10^{-12}–10^{-4} M) for 24 hours. β-Actin is used as the loading control. **B,** Effect of dasatinib (10^{-12}–10^{-4} M) on apoptosis in the c-Kit mutant HBL cells, 48 hours after treatment. Data are presented as means ± SEM ($n = 3$ experiments) compared with untreated cells (CTR). Significance was evaluated by ANOVA test $P < 0.001$. **C,** Cells with acquired resistance to dasatinib termed HBL-R were generated by chronic exposure of a sensitive line (HBL) to gradually increasing concentrations of dasatinib (from 10^{-11} to 10^{-6} M) over a period of 12 weeks to reach (**D**) full resistance at any dasatinib concentration tested. Data represent means ± SEM of 3 independent experiments. **E,** Western blot analysis of expression and phosphorylation of c-Kit, ERK, AKT, MITF, and Bcl-2 in HBL and HBL-R cells under specific concentrations of dasatinib (10^{-8} and/or 10^{-6} M) for 24 hours. β-Actin expression is used as the loading control.

the manufacturer's protocol (CRISPR KO Transfection Protocol; Santa Cruz Biotechnologies). Briefly, cells were seeded (3×10^5 cells/well) in 6-well plates 24 hours before transfection, cotransfected with Bcl-2 CRISPR/Cas9 KO or control CRISPR/Cas9 and Bcl-2 HDR plasmids. The efficiency of transfection was monitored by red fluorescence microscopy. Then selection of transfected cells was done by adding the puromycin antibiotic to the culture medium at 2 μg/mL according to the manufacturer's protocol (Santa Cruz Biotechnologies). Bcl-2 KO was then evaluated by Western blotting.

Statistical analysis

IC$_{50}$ values were calculated using GraphPad Prism software (GraphPad Software). Data are expressed as means $\pm$ SEM of at least three independent experiments. Significance $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ were calculated by Student t test and one way ANOVA.

Results

c-Kit mutation/amplification predicts melanoma sensitivity to Dasatinib

First, we screened several melanoma cell lines ($n = 19$) for their sensitivity to dasatinib regarding to their mutation status. **Figure 1A** shows representative dose–response curves. We could distinguish three groups of melanoma lines: (i) highly sensitive to dasatinib with IC$_{50}$ between 10^{-11} and 10^{-9} M, which are all wild-type (WT) for BRAF and NRAS but present either a c-Kit mutation or amplification, (ii) moderately sensitive to dasatinib with IC$_{50}$ about 10^{-8} to 10^{-7} M, and (iii) resistant lines with IC$_{50}$ ranging from 10^{-5} to 10^{-4} M (**Table 1**). The last two groups do not

present any c-Kit alteration but mainly harbour BRAF or NRAS mutations.

To assess the difference at the protein level of each of the three groups, we evaluated the constitutive level of phosphorylation of c-Kit and SRC, which are two known main targets of dasatinib, along with the phosphorylated ERK (MAPK pathway) and AKT (PI3K/AKT pathway) involved in cell proliferation and survival, respectively. As documented in **Fig. 1B**, all sensitive cell lines exhibited high phosphorylation levels of c-Kit whereas it was absent in all other lines. Moderately sensitive cells have high levels of SRC phosphorylation but no association between the sensitivity to dasatinib and the phosphorylation of ERK or AKT could be found. Taken together and in agreement with a previous clinical trial, the presence of genetic c-Kit aberrations anticipate the response to c-kit inhibitors (30). However, we noticed a biphasic profile of sensitive melanoma cells that becomes resistant to the treatment under relatively high doses that could be detected in melanoma patient's blood. It could be a mechanism triggered under specific concentrations of dasatinib and attenuate melanoma response to the drug. For this reason, and after specification of the category of melanoma that could be sensitive to dasatinib, we designed this study to underline the mechanism triggered in sensitive cells under relatively high doses that could explain the inconsistence between preclinical and clinical results.

Effect of dasatinib on key protein expression and cell apoptosis

Dasatinib was reported as oral potent ATP—competitive inhibitor of c-Kit (31). To identify the function and potential effect of dasatinib, we evaluated its effect on MAPK and PI3K pathways in two sensitive cells HBL (^{D820Y}c-Kit; **Fig. 2A**) and LND1 (^{amp}c-Kit; Supplementary Fig. S1A) compared with two resistant cells MM074

Table 1. Genetic alterations of different melanoma cell lines and its association with sensitivity to dasatinib.

Group	Cell line	Melanoma type[a]	Metastatic site[b]	Dasatinib log IC$_{50}$[c]	BRAF mutation status[d]	NRAS mutation status[d]	c-Kit mutation status[d]	Relative c-Kit expression[e]
Highly sensitive	HBL	ALM	LN	−11	WT	WT	**D820Y**	1.4
	LND1	ALM	LN	−9	WT	WT	**Amp**	5.9
	MM028	?	SK	−10	WT	WT	**D816Y**	1.9
	M230	?	?	−9	WT	WT	**L576P**	2.2
Moderately sensitive	MM029	SSM	LN	−8	**V600K**	WT	WT	bd
	MM057	?	LN	−8	WT	**Q61L**	WT	bd
	MM050	SSM	LN	−7	**V600E**	WT	WT	bd
	MM104	Mucosal	LN	−7	WT	WT	WT	bd
	MM125	SSM	LN	−7	WT	**Q61R**	WT	bd
Resistant	MM031	NM	Bladder	−5	WT	WT	WT	0.3
	MM032	SSM	SK	−5	**V600E**	WT	WT	bd
	MM043	SSM	Intestine	−5	**V600E**	WT	WT	0.1
	MM054	?	SK	−5	**V600E**	WT	WT	bd
	MM070	SSM	LN	−5	**V600E**	WT	WT	bd
	MM074	SSM	LN	−5	**V600E**	WT	WT	0.1
	MM116	?	SK	−5	WT	WT	WT	bd
	MM034	?	LN	−4	**V600E**	WT	WT	bd
	MM052	SSM	SK	−4	WT	**Q61R**	WT	bd
	MM133	LMM	LN	−4	**V600K**	WT	WT	bd

[a]Type of primary melanoma: ALM, acral lentiginous melanoma; SSM, superficial spreading melanoma; NM, nodular melanoma; LMM, lentigo maligna melanoma; ?, Unknown primary.
[b]LN, lymph node; SK, skin/cutaneous metastasis.
[c]Evaluation of IC$_{50}$ average for dasatinib by crystal violet assay.
[d]WT, wild-type; Amp, amplification.
[e]Relative c-Kit protein expression (c-Kit/β-actin).

(^{V600E}BRAF; **Fig. 2A**) and MM057 (^{Q61L}NRAS; Supplementary Fig. S1A). Importantly, dasatinib inhibited the phosphorylation of c-Kit, ERK1/2, and AKT only in sensitive cells (**Fig. 2A**; Supplementary Fig. S1A).

Second, as treatment of c-Kit mutant/amplified melanoma cells with dasatinib resulted in a dose-dependent decrease in cell proliferation (**Fig. 1**), we examined its effect on cell apoptosis. We showed that the decrease in cell proliferation could be explained by a dose-dependent induction of apoptosis in HBL (**Fig. 2B**) and LND1 (Supplementary Fig. S1B). However, our initial observations showed a resistance to the inhibition of cell proliferation in the sensitive cell lines (HBL, LND1, and M230) at high dasatinib concentrations (10^{-7}–10^{-5} M) followed by a complete inhibition of cell proliferation at 10^{-4} M (**Fig. 1**). Consequently, the induction of apoptosis was also lower between 10^{-8} and 10^{-5} M in HBL (**Fig. 2B**) as well as in LND1 (between 10^{-7} and 10^{-5} M; Supplementary Fig. S1B), which is of clinical importance because it corresponds to dasatinib doses that may be achieved in melanoma patients blood (32). This resistance is of particular importance due to the capacity of dasatinib to favor a complete inhibition of MAPK and PI3K pathways under all concentrations indicating that some mechanisms of resistance may be triggered by dasatinib itself at high concentrations. On the other hand, HBL-R cells with acquired resistance to dasatinib revealed a 500-fold increase in IC$_{50}$ to the drug (**Fig. 2C** and **D**). Treatment of HBL-R cells with dasatinib still able to inhibit c-Kit phosphorylation and downstream signalling pathways (**Fig. 2E**). These indicate that early and developed acquired resistance to dasatinib are independent of reactivation of MAPK and PI3K/AKT pathways associated with most of resistance mechanism to MAPK inhibitors in melanoma (pERK/ERK: 0.096 and 0.06, pAKT/AKT: 0.006 and 0.005 fold change under 10^{-8} and 10^{-6} of dasatinib, respectively; pERK/ERK: 0.69 and 0.16, pAKT/AKT: 0.021 and 0.02 fold change of untreated HBL-R and HBL-R treated with dasatinib, respectively; **Fig. 2E**). Protein expression (fold change) are calculated in reference to β-actin and then normalized by the control protein (untreated parental cells). As MITF is a central transcription factor in melanocytes and melanoma, and as we previously reported that MITF/Bcl-2 pathway can be associated with drug resistance in N-RAS-mutant melanoma (26), we investigated the same mechanism under different dasatinib concentrations, screening active and less active concentrations. Notably, MITF consists of at least five isoforms, MITF-A, MITF-B, MITF-C, MITF-H, and MITF-M, varies at their N-termini and expression patterns. MITF-M, is the first identified, expressed exclusively in melanocytes and melanoma and consequently evaluated in our work (33, 34). We found significant increase of both MITF and Bcl-2 expression at drug concentrations as low as 10^{-8} M peaking at 10^{-6} M in HBL (MITF: 3.2 and 2.1, Bcl-2: 4- and 7-fold change respectively; **Fig. 2E**) and LND1 (MITF: 2.1 and 3.8, Bcl-2: 1.3- and 1.9-fold change, respectively; Supplementary Fig. S1C). This finding is highly supported in HBL-R cells were both MITF and Bcl-2 are strongly expressed at the basal level or after treatment with the drug (MITF: 2.2 and 2.3, Bcl-2: 10- and 13-fold change, respectively; **Fig. 2E**).

Dasatinib stimulates MITF/Bcl-2 in melanoma through CRTC3 pathway

MITF activity is stimulated by two different pathways, either by the MSH/MC1R/PKA/CRTC/CREB (GPCR signaling) or c-Kit signaling pathway (16, 35). We wondered the mechanism by which dasatinib upregulates MITF expression. As dasatinib completely shut down MAPK and PI3K pathways in HBL, LND1, and HBL-R cells, MITF activation may return to the first pathway. MITF, is a central transcription factor in melanocytes and the cAMP/MITF pathway is a key regulator of pigmentation (main function of melanocyte) and is involved in the regulation of survival and cell fate of melanoma cells (24, 36). The effect of α-MSH that drives melanogenesis (pigmentation) through the cAMP/PKA/CRTC/CREB/MITF pathway is already well documented. The binding of α-MSH to MC1R boosts cAMP production, which triggers the PKA-mediated phosphorylation and inactivation of SIK proteins, leading to the dephosphorylation and nuclear entry of the CRTCs, which bind to CREB over relevant promoters, stimulate MITF, and consequently induce pigmentation (21, 37–39). Recently, it was shown that treatment with dasatinib or bosutinib inhibit SIK2, resulting in the inhibition of CRTC3 phosphorylation, activating CREB gene expression such as MITF. Nowadays, small molecule SIK inhibitors, to stimulate MITF and melanogenesis, are proposed for safe skin tanning (22, 23).

To study the regulation of the cAMP/CRTC/CREB/MITF axis, the signaling cascade needs to be stimulated to trigger the transcriptional activity of CREB. This can be accomplished by using FSK (through a direct activation of adenylate cyclase) to favor CRTC dephosphorylation and consequently CREB-mediated gene expression such as MITF (19, 40). In this context, we used the FSK to study the activation of MITF through the CRTC/CREB pathway under dasatinib treatment (**Fig. 3**), and to investigate the involvement of MITF in the resistance of melanoma cells to the drug (**Fig. 4**). Dasatinib or FSK treatment alone resulted in an increase of MITF, tyrosinase, and TYRP-1 mRNA levels. This increase is more prominent and conspicuous with a treatment combining dasatinib and FSK (**Fig. 3A**). As described in the literature, we found that FSK treatment in our cells (HBL) resulted in SIK inhibition revealed by CRTC3 dephosphorylation, CREB activation through MITF synthesis, and pigmentation without any modulations in MAPK or PI3K/AKT pathways (Supplementary Fig. S2; ref. 19). Otherwise, dasatinib treatment in melanoma (HBL) turns off both MAPK and PI3K/AKT pathways from one side (**Fig. 3B**; pERK/ERK: 0.26 and 0.24, pAKT/AKT: 0.06- and 0.04-fold change under 10^{-8} and 10^{-6} M of dasatinib, respectively), but in the other side, and as reported previously (22, 23), results in a SIK inhibition as documented by the decrease in CRTC3 phosphorylation and CREB-induced gene expression such as MITF and its downstream targets (MITF: 2.5 and 5.6, tyrosinase: 1.5 and 2, TYRP-1: 4 and 5, Bcl-2: 4- and 6-fold change under 10^{-8} and 10^{-6} M of dasatinib, respectively; **Fig. 3B**). Interestingly, KO of SIK2 gene favor an upregulation of the basal level of MITF (3.2-fold change) and Bcl-2 (1.5-fold change) genes in HBL KO SIK compared with HBL SCR (**Fig. 3C**). Furthermore, MITF activity following dasatinib treatment mediated by the CRTC3 pathway is shown by the increases of tyrosinase and TYRP-1 expression as well as pigmentation (**Fig. 3B** and **D**). Coherently, HBL-R cells with acquired resistance to dasatinib showed decreased levels of pCRTC3, high basal level of MITF (4.8-fold change compared with parental cells), TYRP-1 (4.6-fold change compared with parental cells), and consequently pigmentation. The same effect is more pronounced in HBL-R following dasatinib treatment (MITF: 5, tyrosinase: 2, TYRP-1: 6, and Bcl-2: 6-fold change compared with parental cells), which completely turn off the MAPK and PI3K/AKT pathways (pERK/ERK: 0.5 and 0.3, pAKT/AKT: 1.4- and 0.08-fold change in HBL R and HBL R under 10^{-6} M of dasatinib, respectively; **Fig. 3B**). Notably, treatment with dasatinib and FSK results in significant upregulation of CRTC3/MITF/Bcl-2 pathway compared with dasatinib alone or FSK alone (Supplementary Fig. S2). Taken all together, these indicate that dasatinib treatment increases MITF and its downstream Bcl-2 expression by the CRTC3 pathway in melanoma under clinically relevant doses.
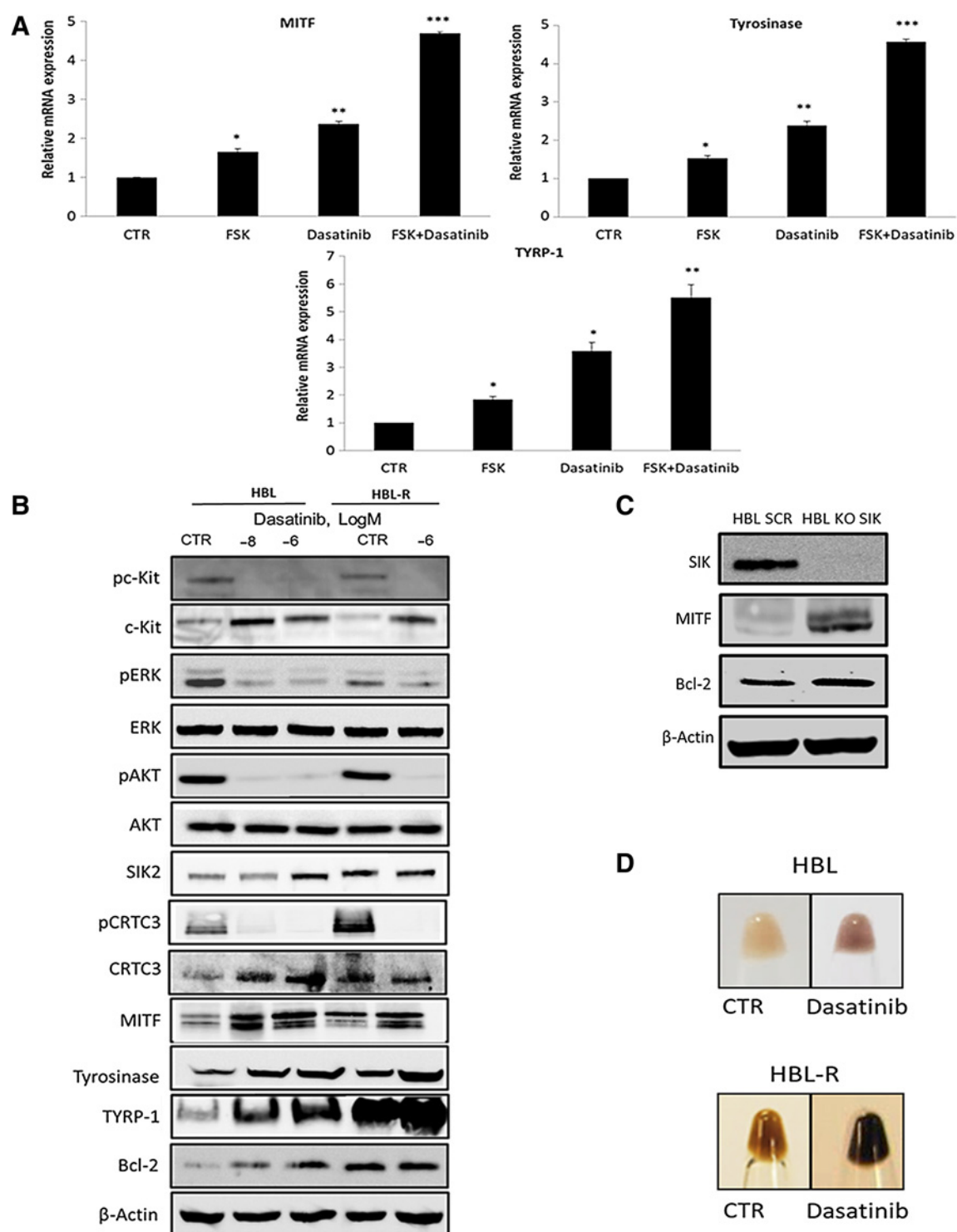
Figure 3.
Dasatinib upregulates MITF and Bcl-2 expression through CRTC3 pathway activation. **A,** Effect of dasatinib (10^{-6} M) and/or FSK (10^{-5} M) on mRNA levels of MITF, tyrosinase, and TYRP-1 in HBL cells. Western blot analysis in (**B**) HBL and HBL-R with/without dasatinib, (**C**) HBL SCR and HBL R KO SIK, β-Actin expression used as loading control. **D,** Cell pellets of melanoma cells (HBL and HBL-R), 3 days after treatment with control vehicle or 10^{-6} M dasatinib.
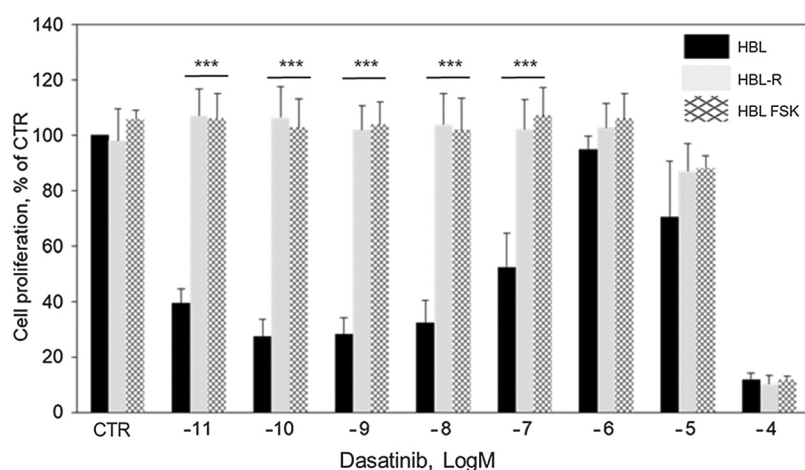
Figure 4.
cAMP stimulation in c-Kit mutant melanoma cells treated with dasatinib exhibited similar profile as with cells with acquired resistance to dasatinib. Comparative effect of dasatinib (10^{-12}–10^{-4} M) on cell proliferation in HBL-R (cells with acquired resistance dasatinib), and parental line (HBL) treated or not by 10^{-5} M FSK, 3 days after treatment. Data are presented as means $\pm$ SEM ($n = 3$ experiments) compared with untreated cells (CTR). Significance was evaluated by two-tailed ANOVA test $P < 0.001$.

MITF/Bcl-2 activation is associated with resistance to dasatinib in c-Kit mutant/amplified melanoma cells

To support and validate our previous observations about the involvement of MITF and Bcl-2 in the resistance of melanoma cells to dasatinib, we conducted a series of experiments aiming at stimulating or inhibiting MITF/Bcl-2 pathway. Cyclic AMP stimulation is a known way to activate MITF and can be achieved by FSK. Like the chronic exposure of c-Kit mutant melanoma cells to dasatinib (HBL-R), the treatment with FSK (used at effective and nontoxic concentration, 10^{-5} M) rendered c-Kit mutant cells (HBL) more resistant to dasatinib compared with parental cells (**Fig. 4**). Pharmacologic activation of cAMP and subsequently MITF by FSK decreased the sensitivity of melanoma cells to dasatinib. These indicate that upregulated MITF expression observed under gradually increasing concentrations of dasatinib confers resistance of melanoma cells to the drug. In the next step, we treated cells with a selective Bcl-2 inhibitor, ABT-199 (used at effective and non-toxic concentration; 1 µmol/L) in combination with dasatinib, and we observed that c-Kit mutant cells (HBL) recovered partial sensitivity to dasatinib (**Fig. 5A**). Moreover, the percentage of apoptotic cells increased up to 37% under dasatinib and ABT-199 versus 14% for dasatinib alone in HBL cells (**Fig. 5B**). Even in c-Kit amplified melanoma cells (LND1), treatment with ABT-199 sensitizes the cancer cells to dasatinib (**Fig. 5C**). More importantly, HBL-R cells were evaluated for their sensitivity to a combination of dasatinib (10^{-6} M) and ABT-199 (1 µmol/L). ABT-199 restored the sensitivity to dasatinib by inhibiting proliferation and enhancing apoptosis in cells with acquired resistance. Indeed, cell proliferation decreased by 50% and cell apoptosis increased by 45% in HBL-R cells exposed to dasatinib and ABT-199 combination compared with dasatinib alone (**Fig. 5D**). It is worth noting that this combination shows more pronounced effects in cells with acquired resistance as compared with parental cells. This observation can be related to the higher basal level of both MITF and Bcl-2 in resistant cells (HBL-R) compared with parental ones (HBL; compare **Fig. 5A**, **B**, and **D**).

KO of Bcl-2 moderates the intrinsic resistance occurring at high dasatinib concentrations

To validate our data, we performed a specific disruption of Bcl-2 gene through CRISPER/Cas9 technology. Then, in Bcl-2 KO cells (HBL KO Bcl-2), we first checked the absence of Bcl-2 expression by Western blot analysis and observed that its expression was no further stimulated by 10^{-6} M dasatinib (**Fig. 6A**). In agreement with our previous data (**Fig. 3**), the same treatment applied to HBL SCR

cells increased both MITF and Bcl-2 expression but inhibited c-Kit, ERK, and AKT phosphorylation (**Fig. 6A**). Of note, Bcl-2 KO weakly affected cell proliferation (data not shown). Likewise, we evaluated cell proliferation and apoptosis under dasatinib and found that, unlike HBL SCR cells, HBL KO Bcl-2 cells recovered sensitivity to dasatinib at concentrations beyond 10^{-7} M at which intrinsic resistance is observed (**Fig. 6B**) and showed more than two-fold increases of apoptotic cells as compared with HBL SCR cells under 10^{-6} M dasatinib (**Fig. 6C**). Interestingly and coherently, KO of Bcl-2 in HBL-R cells (Supplementary Fig. S3A) sensitizes the line to dasatinib by increasing apoptosis but not in HBL-R SCR that remains completely resistant to dasatinib (Supplementary Fig. S3B).

Discussion

c-Kit is an important target particularly in acral and mucosal melanoma where mutations of this gene are frequent. On the basis of clinical trials, most of melanoma responders shows mutation on exon 11 or 13, harboring inactive conformation of c-Kit at which clinically approved drugs such as imatinib and nilotinib could bind (41). Patients carrying c-Kit mutations in exons 17–18, particularly at A-loop domain, shows active conformation of c-Kit at which most of RTK inhibitors could not bind and patients achieved stable disease (42). Indeed, *in vitro* studies suggested potential use of dasatinib in patients with melanoma but this did not translate into any clinical benefits (15), despite that, among RTK inhibitors, dasatinib is able to recognize both active and inactive conformation of c-Kit.

Surprisingly and unexpectedly, dasatinib was less effective at high concentrations (µmol/L) than at lower ones (nmol/L) in all lines with c-Kit alterations (mutation or amplification) in terms of inhibition of cell proliferation and induction of apoptosis. As such concentrations have been reported to be relevant in melanoma patient blood (32), our observation of an early resistance at high dasatinib concentrations became of importance and deserved further investigation. In fact, some results from small phase II studies could be more encouraging than previously thought (15).

Broadly, many mechanisms by which kinase-driven cancers develop intrinsic and/or acquired resistance associated with tyrosine kinase inhibitors has been described (43). Point mutations in the target gene can change the binding potential of the inhibitor through structure alterations which may constitute a main mechanism of resistance to such class of drugs. It was reported that resistance developed after prolonged exposure to c-Kit inhibitors in melanoma was associated
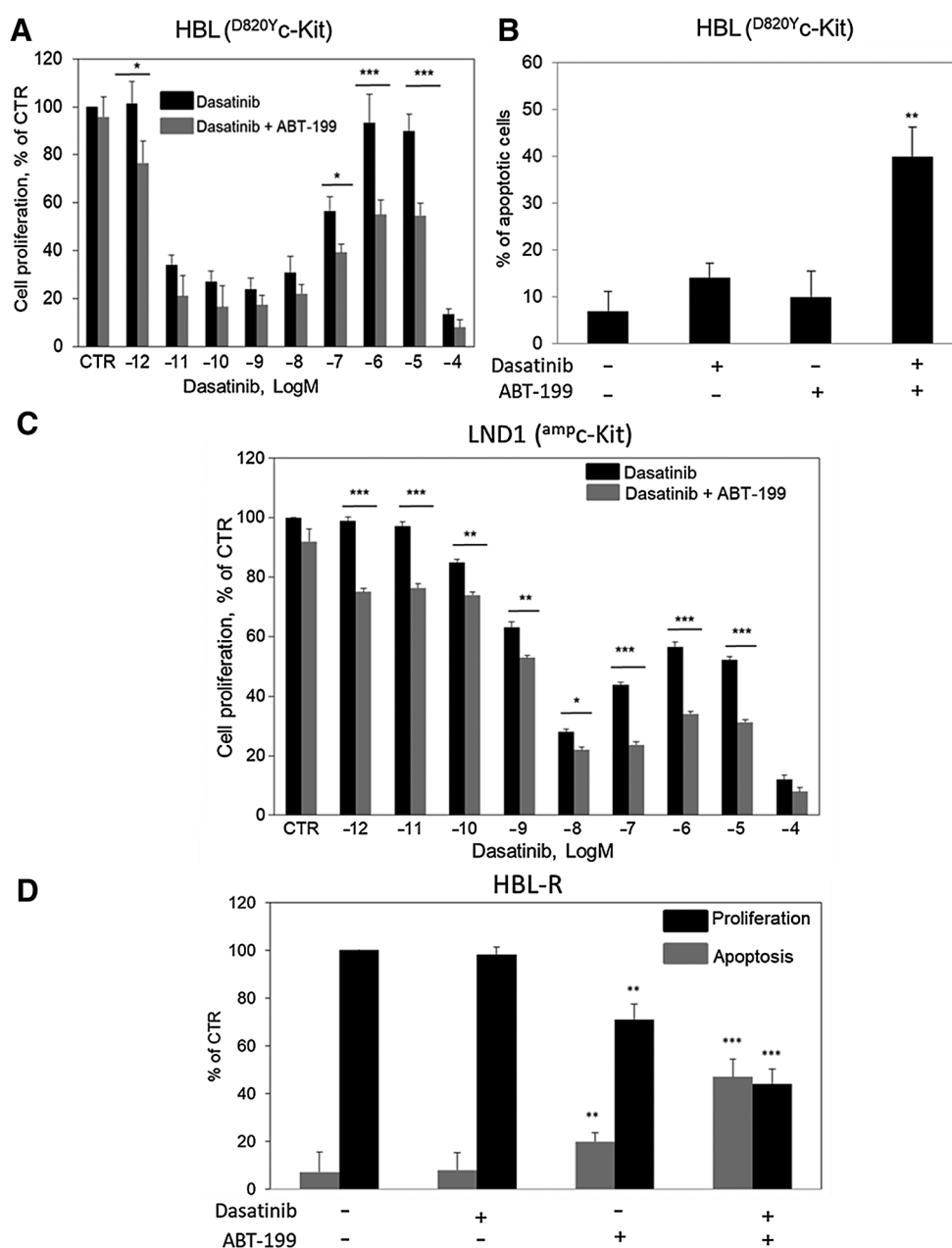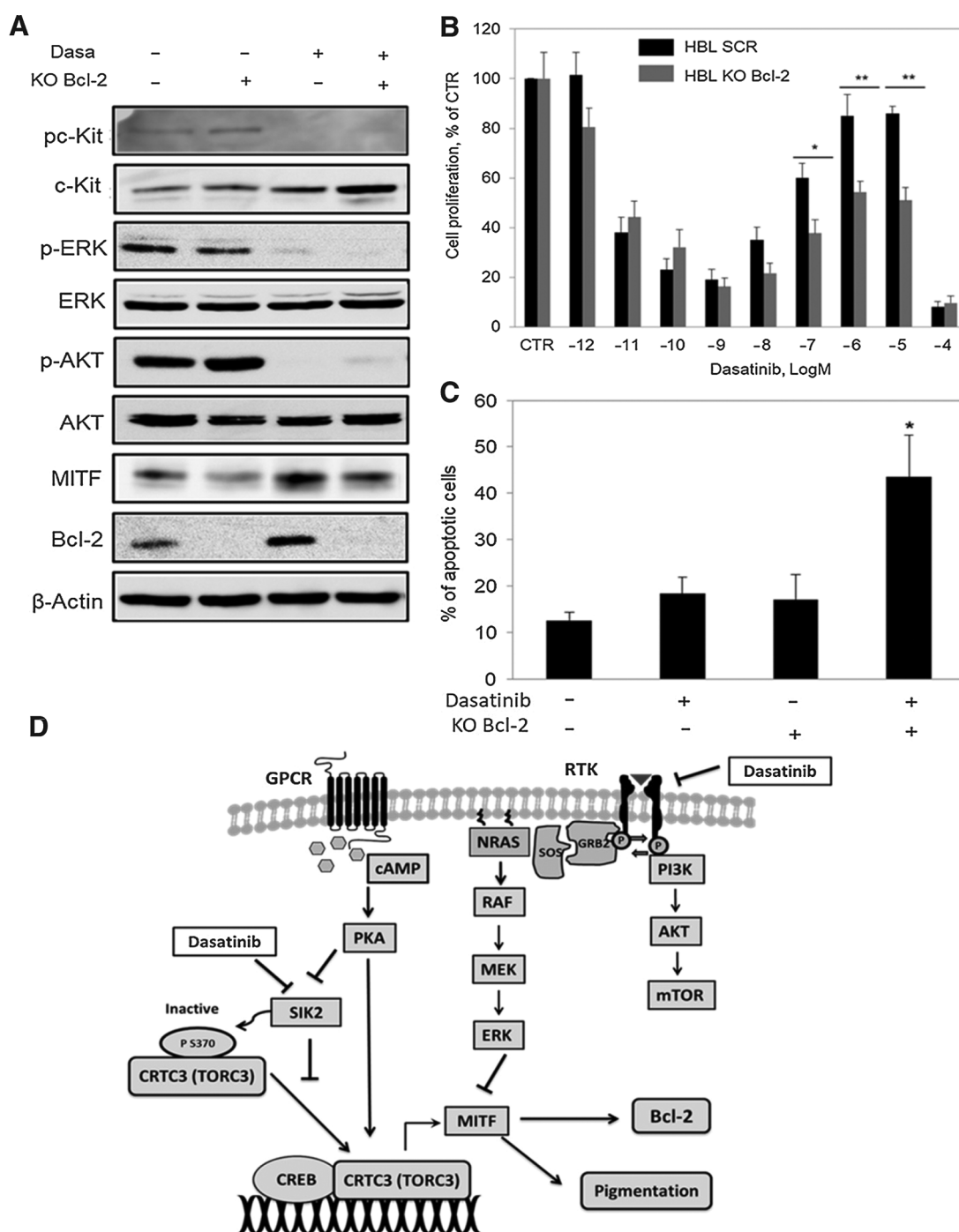
Figure 5.
Bcl-2 inhibition sensitizes melanoma cells to dasatinib. **A,** Effect of Bcl-2 specific inhibition by 1 μmol/L ABT-199 on c-Kit mutant melanoma cells (HBL) treated with dasatinib. **B,** Evaluation of the percentage of apoptotic cells in HBL melanoma cells under dasatinib (10^{-6} M) and/or ABT-199 (1 μmol/L). **C,** Effect of dasatinib (10^{-12}–10^{-4} M) and/or ABT-199 (1 μmol/L) on cell proliferation of LND1 (c-Kit amplified melanoma cells). Data are presented as means ±SEM ($n = 3$) compared with cells treated with dasatinib alone. **D,** Effect of dasatinib (10^{-6} M) and/or ABT-199 (1 μmol/L) on cell proliferation and apoptosis of HBL-R. Data are presented as means ± SEM ($n = 6$) compared with cells treated with dasatinib.

with reactivation of c-Kit signaling due to secondary c-Kit mutations and a switch to an alternative c-Kit inhibitor or targeting the MAPK/PI3K-AKT signaling were proposed as therapeutic strategies (44). Unfortunately, such combinations were not validated clinically and, consequently, the molecular mechanisms underlying a resistance to RTK inhibitors appear to be more complex and additional non-mutational mechanisms have been documented in recent years (45). Therefore, we set a series of experiments to have a detailed close look

into the mechanism by which dasatinib can be less effective at clinically relevant concentrations.

First, due to the importance of patient selection that can respond to critical treatment, we evaluated cell proliferation under dasatinib in a series of melanoma lines. In accordance with clinical trials which reported effect of RTK inhibitors in selection of patients harboring c-Kit aberrations (46, 47), we found that responses to dasatinib is limited to melanoma cells harboring c-Kit alteration. Second, to evaluate

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Figure 6.
Knockout of Bcl-2 moderates resistance to dasatinib occurring at relatively high dasatinib concentrations. **A,** Western blot analysis of c-Kit, ERK, and AKT, also MITF and Bcl-2 in scramble HBL (transfected with control vector) compared with Bcl-2 KO HBL cells with/without dasatinib. β-Actin expression is used as the loading control. **B,** Effect of dasatinib (10^{-12}–10^{-4} M) on the proliferation of both melanoma cell lines (HBL SCR and Bcl-2 KO HBL). **C,** Effect of dasatinib (10^{-6} M) on apoptosis in HBL SCR and HBL KO Bcl-2 cells, 3 days after treatment. Data are presented as means ± SEM ($n = 6$) compared with HBL SCR. **D,** Simplified summary scheme showing the involvement of the CRTC3/MITF/Bcl-2 pathway in the resistance of c-Kit mutant melanoma to dasatinib. Dasatinib inhibits both MAPK/PI3K-AKT pathway and SIK2 that reduce CRTC3 phosphorylation enabling the latter to translocate into the nucleus to promote CREB target gene expression such as MITF and, consequently, its downstream targets including Bcl-2 as well as the pigmentation machinery.

dasatinib effect in sensitive cells, we compared lines harboring either c-Kit alterations to lines with BRAF or NRAS mutation under doubling concentrations of dasatinib and found a significant phosphorylation inhibition of c-Kit, ERK, and AKT associated with c-Kit mutation. Strikingly, all inhibitions of phosphorylation occurred at low (nmol/L) and high (μmol/L) concentrations but the proliferation of melanoma cells was significantly restored and, consequently, the apoptosis was reduced at high doses ranging from 10^{-8} to 10^{-5} M. A closer look at these concentrations revealed the stimulation of both MITF and Bcl-2 expression that can be picked-up already at 10^{-8} M. In addition, cells with acquired resistance to dasatinib (HBL-R) showed an increase in MITF and Bcl-2 levels. We relied on previous observations indicating that MITF mRNA levels correlate with CRTC3 expression, and we wondered if this upregulation of MITF following dasatinib treatment is caused by CRTC/CREB/MITF pathway activation (48). Moreover, dasatinib was developed as an RTK inhibitor but it was described with ability to induces macrophage polarization through inhibition of the salt-inducible kinases SIK2, what causes CRTCs dephosphorylation and resulting in CREB gene expression (22, 23). Accordingly, to validate the activation of MITF by CRTC3 pathway under dasatinib and its involvement in such resistance in sensitive cells, we used the FSK to stimulate the production of cAMP to activate the CRTC/CREB/MITF signaling pathway (49, 50). Both FSK and 1 μmol/L dasatinib treatment in melanoma cells resulted in an increases in mRNA levels of MITF, tyrosinase, and TYRP-1. Interestingly, this upregulation is more pronounced under dasatinib and FSK compared with each effector alone. FSK treatment resulted in SIK inhibition revealed by dephosphorylation of CRTC3 and induced the CREB/MITF/TYR/TYRP-1-mediated pigmentation. Furthermore, like FSK, dasatinib causes SIK inhibition, CRTC3 dephosphorylation, and CREB-induced gene activation leading to an increase in MITF, tyrosinase, and TYRP-1 protein levels. Beside, overexpression of MITF by FSK in HBL resulted in a dose-independent resistance to dasatinib. Taken together, this indicates that dasatinib, under increasing concentrations, up-regulates MITF expression by CRTC3/CREB pathway conferring resistance of melanoma cells to the treatment. Importantly, the upregulation of MITF pathway following dasatinib treatment can regulate cell survival by promoting the anti-apoptotic factor Bcl-2 (27). Particular attention was addressed to other anti-apoptotic proteins such BCL2A1, Bcl-XL, and BIRC7. Only Bcl-2 was found upregulated under dasatinib (Supplementary Fig. S4). To evaluate the role of Bcl-2 in such resistance, we altered Bcl-2 activity by ABT-199, a known selective inhibitor, or by KO strategies. ABT-199 is a potential selective inhibitor of pro-apoptotic factor Bcl-2, which causes Bax/Bak-mediated apoptosis that is principally triggered by the initiator BH3-only protein Bim (51). As expected, our cells with intrinsic or acquired resistance to dasatinib became sensitive to dasatinib after exposure to ABT-199 and this combination acted in synergy to inhibit cell proliferation and induced apoptosis. Notably, this effect is more pronounced in cells with acquired resistance (HBL-R) compared with parental ones. An explanation can be that HBL-R cells are more dependent on MITF/Bcl-2 pathway for their survival. Coherently, Bcl-2 KO in HBL resulted in a significant decrease of cell proliferation and promotion of apoptosis indicating that Bcl-2 protein can confer resistance to apoptosis in melanoma. Of note, KO of Bcl-2 using a different sgRNA also resulted in slight decrease in MITF expression that we attributed to a negative feedback following Bcl-2 gene loss especially that Bcl-2 is mainly regulated by MITF (data not shown; ref. 27). Hence, Bcl-2 inhibition or KO provides evidences for Bcl-2 activation and its involvement in the early resistance of melanoma cells to dasatinib, and indicates that Bcl-2 expression is essential to maintain cell survival despite the inhibition of both MAPK and PI3K/AKT pathways.

As a prospect, the validation of our findings in patient tissues seemed the next obvious step to perform. Unfortunately, it is rather very difficult to obtain either TCGA data or tissue biopsies from c-Kit patients with melanoma and even less before and under dasatinib. However, this is a first report putting forward a peculiar mechanism of resistance to an RTK inhibitor not associated to a secondary c-Kit mutation and contrasting with previous preclinical findings attributing the resistance to a secondary c-Kit mutation that was not observed in c-Kit-treated patients with melanoma.

Conclusions

In conclusion, the high efficacy of dasatinib alone as targeted therapy against altered c-Kit in melanoma cell lines shows moderate to mediocre efficacy in patients with melanoma despite that, among clinically approved RTK inhibitors, it has unique features as it targets more kinases and binds both of active and inactive conformation of c-Kit. In this context, we identified the CRTC3/MITF/Bcl-2 pathway as an important mechanism of intrinsic and acquired resistance to dasatinib in melanoma, which can be stimulated by dasatinib itself and may have contributed, at least in part, to the clinical inefficacy of this drug as presented in **Fig. 6D**. Our observation also support that any cAMP stimulation may elicit the same effect such as the one produced by corticosteroids (prednisone), which is often prescribed with chemotherapy, and may have contributed to the ineffectiveness of dasatinib as well.

Authors' Disclosures

W. Miller reports grants from CIHR, Cancer Research Society, and grants and personal fees from BMS during the conduct of the study; grants and personal fees from MERck, personal fees from Novartis, Pfizer, Mylan, Sanofi, and Roche outside the submitted work. A. Awada reports grants from Roche, BMS, personal fees from Lilly, Amgen, ESAI, Pfizer, Novartis, Genomic Health, Ipsen, Bayer, Leo Pharma, Merck, Daiichi, and Seattle Genetics outside the submitted work. No disclosures were reported by the other authors.

Authors' Contributions

M. Sabbah: Conceptualization, resources, software, validation, investigation, writing–original draft, project administration, writing–review and editing. **M. Krayem:** Conceptualization, data curation, validation, methodology. **A. Najem:** Data curation, validation, visualization, methodology. **F. Sales:** Resources, project administration. **W. Miller:** Validation, methodology. **S. del Rincon:** Validation, methodology. **A. Awada:** Resources. **G.E. Ghanem:** Conceptualization, resources, data curation, software, supervision, funding acquisition, validation, writing–review and editing. **F. Journe:** Conceptualization, resources, funding acquisition, writing–review and editing.

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Dasatinib Stimulates Its Own Mechanism of Resistance by Activating a CRTC3/MITF/Bcl-2 Pathway in Melanoma with Mutant or Amplified c-Kit

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