The T197A knock-in model of Cdkn1b gene to study the effects of p27 restoration in vivo.

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ABSTRACT

The CDK inhibitor, p27kip1, encoded by the Cdkn1b gene can negatively modulate cell proliferation. The control of p27 activity during the cell cycle is regulated at multiple levels including transcription, translation and protein stability. The last residue of p27 (threonine 198 in human, threonine 197 in mouse) is involved in the control of protein stability. We have generated a murine knock-in model (Cdkn1bT197A) in which threonine 197 is replaced by alanine, which renders p27 protein highly unstable due to a high rate of proteasomal degradation. Expectedly, Cdkn1bT197A/T197A mice present increased body size and weight, organomegaly and multiple organ hyperplasia, similar to what observed in Cdkn1bKO/KO mice. We investigated the effects exerted by the restoration of normal levels of p27 protein in the tissue of Cdkn1bT197A/T197A mice. We found that proteasome inhibition with Bortezomib rescues the hyperplasia induced by the lack of p27 expression in Cdkn1bT197A/T197A but not in Cdkn1bKO/KO mice. However, BAY 11-7082, a proteasome inhibitor that stabilizes IKB but not p27, fails to rescue hyperplasia in Cdkn1bT197A/T197A mice. Bortezomib increases p27 half-life and reduces the proliferation in MEFs derived from Cdkn1bT197A/T197A but not from Cdkn1bWT/WT mice whereas BAY 11-7082 had no effect on the protein levels of p27 and on the proliferation rate of Cdkn1bT197A/T197A MEFs.

The results presented here demonstrate that Cdkn1bT197A/T197A mice represent an attractive in vivo model to investigate whether the targeting of p27 degradation machinery might prove beneficial in the treatment of a variety of human proliferative disorders caused by increased turnover of p27 protein.
INTRODUCTION

Cell cycle progression is regulated by the activity of cyclin dependent kinases (CDKs) that induce progression towards S phase and initiate mitosis. CDKs are activated by cyclin binding and inhibited by CDK inhibitors (CKI) (1). Two families of CKI negatively regulate cell cycle progression: inhibitors of CDK4 (INK4) and kinase inhibitor proteins (KIP) (1,2). The KIP inhibitor p27Kip1 (hereafter p27) is encoded by the Cdkn1b gene (3-5). p27 can modulate the activities of most CDK complexes (1) thus regulating the progression of cells from G1 to S and from G2 to M. In addition, recent studies have shown that p27 is also capable to regulate, motility and apoptosis (6-8).

Accordingly, Cdkn1bKO/KO mice are characterized by increased body size, organomegaly and hyperplasia (9-11). In addition, Cdkn1bKO/KO mice spontaneously develop pituitary adenomas (9-11) and are more susceptible to tumorigenesis induced by chemical carcinogens and/or irradiation (12-14).

The control of p27 activity during cell cycle is complex and involves regulation at multiple levels including transcription, translation, protein stability, distribution among different cyclin-CDK complexes and cellular localization (3,15). In particular, the intracellular level of p27 is regulated by phosphorylation at threonine 187 (16,17), ubiquitylation and degradation by the 26S proteasome of T187-phosphorylated p27 (18,19).

Recently, the phosphorylation of p27 on its very last residue [threonine 198 (T198) in human, threonine 197 (T197) in mouse] by different kinases (20,21) has also been proposed to control p27 stability (22,23).

In this manuscript we have generated a knock-in mouse model in which T197 of p27 is replaced by an alanine (Cdkn1bT197A) to study the long-term effects of restoring p27 levels in vivo. We observed that, similarly to the phenotype shown by Cdkn1bKO/KO mice, Cdkn1bT197A/T197A mice present increased body size, organomegaly and hyperplasia in multiple organs. However, at difference with what happens with Cdkn1bKO/KO mice, the treatment of Cdkn1bT197A/T197A mice with proteasome inhibitors reduces tissue hyperplasia induced by the lack of p27 expression in vivo and reverts almost completely body size and organomegaly.
MATERIALS AND METHODS

Animal treatment

Animal experimentation was approved by the local ethical committee “Comitato Etico per la Sperimentazione Animale” (CESA) of Biogem, IRGS (Ariano Irpino, Italy) (protocol approval number 43/12) and by Italian Ministry of Health (616/2015-PR). The experimentation was conform to regulations and guidelines of Italy and the European Union. All efforts were made to minimize animal suffering (S1 ARRIVE Checklist). C57BL/6 p27 knock-out (Cdkn1bKO/KO) mice (24) were maintained at CRO animal facility (Aviano, Italy). Animal experimentation related to Cdkn1bKO/KO mice was approved by Italian Ministry of Health (616/2015-PR).

Mice of 3, 6 and 9 months of age were treated twice a week for 4 weeks with 1mg/kg of Velcade® (Bortezomib, hereafter BZB) or 5 mg/kg of (E)3-[(4-methylphenyl)-sulfonyl]-2-propenenitrile (CAS number, 195462–67-7, hereafter BAY 11-7082) (25). BZB for the animal treatment was a gift from Dr. Marco Rossi (University Magna Graecia, Catanzaro). BAY 11-7082 was purchased from Sigma-Aldrich (Saint-Louis, MO, USA).

Histological analysis and immunohistochemistry

Animals were weighed at regular intervals starting from three weeks of age. At necropsy, internal organs were weighed and fixed in 10% neutral buffered formalin (Sigma). Tissues were embedded in paraffin, cut in 5 μm sections and stained with hematoxylin and eosin according to standard methods.

Ki67 immunostaining (1:200, #4203-1 Epitomics, Burlingame, CA) was performed according to the Dako Real Detection System (Dako K5001). At least 20 different fields at a magnification of 20X were analysed for each sample in order to define the proliferation rate.

Preparation of lysates and immunoblotting

Mouse organs were homogenized with Tissue Lyser (Qiagen, The Netherlands, Venlo) in cold lysis buffer (20 mM HEPES, 150 mM NaCl, 10% glycerol, 0.1% Triton X-100) containing protease and phosphatase inhibitors (Sigma Aldrich).

Cells were lysed in NP40 buffer (0.5% NP-40, 50 mM HEPES [pH 7], 250 mM NaCl, 5 mM EDTA, 0.5 mM EGTA [pH 8]) supplemented with protease and phosphatase inhibitors. Immunoblot were carried out by standard methods. Membranes were incubated overnight at +4°C with the following antibodies: anti-p27 (#2552) and anti-IkB (#9242) from Cell Signaling Technology (Denver, MA, USA) and anti-actin (sc-1616) from Santa Cruz Biotechnology (Santa Cruz, CA). Chemiluminescence was detected with Alliance Mini WL2M system (Uvitec, Cambridge, UK).

Densitometric analysis of gel bands was carried out with ImageJ software (NIH, Bethesda, MD).
Trypan Blue exclusion and 5-Bromo-2′-deoxy-uridine (BrdU) incorporation assays

MEFs were established from embryos dissected at 12.5 dpc as described elsewhere (26) and used at passages P1-P5. During treatments, MEFs were plated at a density of 3x10³/well in triplicate and treated every 48h with the indicated compounds. For Trypan Blue exclusion assay, MEFs were harvested and diluted 1:1 with 0.4% Trypan Blue solution and counted with TC20 cell counter (Biorad, Hercules, CA). Experiments were repeated at least three times.

For BrdU incorporation assay, cells were plated onto coverslips in 6-well culture plates in complete DMEM and serum starved for 72 hours. Complete medium with or without drugs was added as indicated. BrdU was added 60 minute prior to processing. Detection of BrdU incorporation was carried out according to manufacturer’s instructions (5-Bromo-2′-deoxy-uridine Labeling and Detection Kit I, Roche Diagnostics) and visualized by microscopy (Axioplan 2, Zeiss). BrdU incorporation was measured by counting positive nuclei in a total of 500 cells in triplicate experiments.

Determination of protein half-life

For determination of protein half-life in exponentially growing MEFs, cells were seeded at ~60% confluency in 100-mm plates, grown over-night and treated with 10 nM BZB for 16 hours. Cycloheximide (10 µg/mL) was added for the indicated time.

Statistical analysis

Continuous variables were analyzed by two tailed Student’s t test, One-way or Two-way ANOVA, as indicated in the text (GraphPad Software, Inc., La Jolla, CA, USA). The statistical significance threshold was set at a P value of 0.05 or less.
RESULTS

**Cdkn1b<sup>T197A/T197A</sup> mice are viable and giant**

The targeting vector to generate the knock-in T197A mouse strain (Cdkn1b<sup>T197A/T197A</sup>) is described in Supplementary Methods and Figure S1A. Germ line transmission of the knocked-in allele was confirmed by PCR (Supplementary Figure S1B-C).

Ten consecutive generations of heterozygous (Cdkn1b<sup>WT/T197A</sup>), homozygous (Cdkn1b<sup>T197A/T197A</sup>) and control (Cdkn1b<sup>WT/WT</sup>) mice were observed up to 12 months of age. During this period mice were fully viable, and only two knock-in mice developed intestinal or mammary tumours diagnosed at necropsy. Cdkn1b<sup>T197A/T197A</sup> mice showed compromised fertility since only 16% of pregnant animals were able to carry pregnancy to term in a cohort of 50 homozygous females.

Cdkn1b<sup>T197A/T197A</sup> mice were significantly larger and heavier in size and weight (38.7% and 30.9% weight increase for females and males, respectively) than control wild type littermates whereas Cdkn1b<sup>WT/T197A</sup> heterozygotes were intermediate. Figure 1A shows growth curves for wild type Cdkn1b<sup>WT/WT</sup>, heterozygous Cdkn1b<sup>WT/T197A</sup> and homozygous Cdkn1b<sup>T197A/T197A</sup> mice (females on the left, males on the right). Differences in weight were not evident at birth and became significant at 2-3 weeks. Weight difference reached maximal values by 10 weeks of age and during adulthood. We found that weight increase was caused by significant enlargement of most internal organs. In Figure 1B the weight values of intestine, uterus and spleen for different cohorts of wild type and/or homozygous Cdkn1b<sup>T197A/T197A</sup> mice are reported. The average weight of the organs resected from homozygous Cdkn1b<sup>T197A/T197A</sup> mice (at 3 months of age) was consistently higher compared with the organs resected from wild type mice (of same age) [intestine: 199.7 (± 29.5) mg vs 332.5 (± 83.6) mg; uterus 100 (± 22) mg vs 145.9 (± 28) mg; spleen: 103.8 (± 27.9) mg vs 211.9 (± 64.8) mg]. Pictures showing representative examples of intestine, uterus and spleen of Cdkn1b<sup>WT/WT</sup> and Cdkn1b<sup>T197A/T197A</sup> mice are reported in Figure 1C.

**Histological analysis of Cdkn1b<sup>T197A/T197A</sup> mice**

Male and female mice of 3 (10 Cdkn1b<sup>WT/WT</sup>, 10 Cdkn1b<sup>T197A/T197A</sup>), 6 (6 Cdkn1b<sup>WT/WT</sup>, 13 Cdkn1b<sup>T197A/T197A</sup>) and 9 months (7 Cdkn1b<sup>WT/WT</sup>, 9 Cdkn1b<sup>T197A/T197A</sup>) were sacrificed to perform histological analysis. Macrosopically, both Cdkn1b<sup>WT/T197A</sup> and Cdkn1b<sup>T197A/T197A</sup> mice showed no significant gross alteration in the tissue architecture except for the size of the organ when compared with wild type littermates. Histo-pathological analysis revealed the presence of hyperplastic lesions in small intestine, spleen, ovary, uterus, testis, thymus, mammary glands and pituitary gland in all Cdkn1b<sup>T197A/T197A</sup> mice analysed. Hyperplastic lesions were apparently caused by an increase in the number of otherwise normal cells since neither dysplastic nor neoplastic lesions inside organs were
found. Representative images of sections stained with haematoxylin and eosin from intestine (left panel), uterus (middle panel) and ovary (right panel) resected from Cdkn1bT197A/T197A mice are shown in Figure 2A. Insets show sections from wild type mice.

By use of Ki67 and/or TUNEL we investigated whether the observed hyperplastic lesions were the consequence of increased proliferation and/or decreased apoptosis. We found that in control mice the average number of Ki67-positive nuclei was significantly lower than in Cdkn1bT197A/T197A mice (Figure 2B-C). Conversely, no significant difference was observed in the two groups for the staining of TUNEL (Supplementary Figures S2).

These results suggest that the hyperplastic lesions in the organs of Cdkn1bT197A/T197A mice are proliferative.

**BZB increases p27 levels and restores normal size in multiple organs**

Since stability of p27 protein is controlled by the rate of its degradation through the ubiquitine/proteasome pathway, we investigated whether BZB, a proteasome inhibitor currently used in multiple myeloma and other cancers (27), increased the half-life of p27T197A protein, thus restoring normal level in mouse tissues. Wild type and Cdkn1bT197A/T197A mice (males and/or females of 3 months of age) were treated with 1 mg/kg of BZB or with solvent alone and sacrificed after 4 weeks.

Throughout the treatment all mice under analysis were weighed. Results are reported in Figure 3A. Treatment with BZB had no apparent effect of the whole weight of wild type mice (n=10) whereas it induced a significant (p=0.005) reduction in the average weight of Cdkn1bT197A/T197A mice (n=10). After 4 weeks of treatment the average weight of solvent-treated Cdkn1bT197A/T197A mice was 33.2 (± 2.9) g whereas the average weight of BZB-treated Cdkn1bT197A/T197A mice was reduced by approximately 11% (29.46 ± 2.3 g).

We also determined the effects exerted by BZB on tissue size and tissue architecture of mice treated with solvent or BZB. At difference with wild type littermates in which BZB apparently showed no effect, treatment of Cdkn1bT197A/T197A mice with BZB induced a dramatic reduction in organ weight. Figure 3 shows the effects exerted by BZB on thymus, uterus and spleen of Cdkn1bWT/WT and Cdkn1bT197A/T197A mice, respectively. As shown, BZB almost restored the weight of multiple organs to normal levels (Figure 3B-D).

The average weight of the uterus in solvent-treated Cdkn1bT197A/T197A mice was 140.4 (± 35.2) mg whereas that of uterus in BZB-treated Cdkn1bT197A/T197A mice was 87.2 (± 36.5) mg. As reference the average weight of uteri of Cdkn1bWT/WT mice was 95 (± 18.1) mg. Similarly, the treatment with BZB induced a reduction of the average weight of thymus from 130.5 (± 33.2) mg to 88.8 (± 20.1) mg and of spleen from 218.6 (± 80.2) mg to 157.5 (± 42.1) mg in Cdkn1bT197A/T197A but not in Cdkn1bWT/WT mice. In this case, the average weight of thymus of Cdkn1bWT/WT mice was 70.2 (±19.7) mg when
treated with solvent and 63 (± 20.1) mg when treated with BZB and that of spleen was 122.5 (± 36.7) mg when treated with solvent and 129 (± 31) mg when treated with BZB. Pictures showing representative example of uterus, thymus and spleen from solvent- or BZB-treated Cdkn1bWT/WT and Cdkn1bT197A/T197A mice, respectively are reported in Figure 3E. Concomitant with the reduction in the weight of multiple organs, treatment of mice with BZB induced a marked increase in the levels of p27 as detected by densitometric analysis of immunoblot in the organs of Cdkn1bT197A/T197A mice but not in Cdkn1bWT/WT mice (Figure 3F). A representative immunoblot showing the results for 3 Cdkn1bWT/WT mice and 3 Cdkn1bT197A/T197A mice is depicted in Figure 3G.

**Histological analysis of Cdkn1bT197A/T197A mice treated with BZB**

In agreement with the macroscopic data, we found signs of hyperplasia in multiple organs including small intestine, mammary gland, ovary, uterus, testis, thymus and pituitary gland in all Cdkn1bT197A/T197A mice but in none of wild type mice. However, we found that the Cdkn1bT197A/T197A mice showed a markedly reduced degree of hyperplasia if previously treated with BZB (n=5). See Figure 4 for representative images.

Subsequently, we used Ki67 staining to investigate whether the observed effects exerted by BZB on the hyperplastic lesions were the consequence of decreased proliferation. We found that the number of Ki67-positive cells was higher in the organs of Cdkn1bT197A/T197A mice compared with the organs of Cdkn1bWT/WT mice. See column V in Figure S3. Conversely, Cdkn1bT197A/T197A mice treated with BZB showed decreased Ki67 staining in all organs examined as compared with mice treated with solvent alone (See columns B and V in Figure S3). Quantitative analysis of Ki67 staining in different organs of Cdkn1bWT/WT and Cdkn1bT197A/T197A mice demonstrated that the number of Ki67-positive nuclei (mean ±SD) was higher in Cdkn1bT197A/T197A mice than in Cdkn1bWT/WT mice (Figure S3E): 3-fold in the small intestine (20±5 vs 6±5) and uterus (25±5 vs 8±4), 5-fold in the testis (20±3 vs 4±2) and 12.5-fold in the thymus (25±3 vs 2±1). The treatment with BZB induced a marked reduction in the number of Ki67-positive cells of 25% (15±5) in the small intestine, 20% (20±5) in the thymus and of 40% in testis (12±4) and uterus (15±5), respectively.

These results suggest that a reduction in cell proliferation, likely dependent on the stabilization of p27 after proteasome inhibition, may account for the observed effects of BZB on the hyperplastic lesions generated in the organs of Cdkn1bT197A/T197A mice.

**BZB increases p27 levels and restores normal weight of multiple organs**

The initial rationale for use of BZB in multiple myeloma was inhibition of NFkB activity as IkB is a substrate of the proteasome (28). However, the intracellular levels of multiple cell cycle-related proteins including p27 are regulated by the proteasome (29). Therefore, we performed two different
set of experiments to further support the evidence that the effects elicited by BZB on the tissues of Cdkn1b<sup>T197A/T197A</sup> mice were mediated by the increase in the levels of p27 protein.

First, to exclude that the anti-hyperplastic effects of BZB in the tissues of Cdkn1b<sup>T197A/T197A</sup> mice were mediated by inhibition of NFκB, we treated wild type (n = 5) and Cdkn1b<sup>T197A/T197A</sup> mice (n = 5) with BAY-11-7082, a compound that stabilizes IkBα thus inhibiting NFκB.

Wild type and Cdkn1b<sup>T197A/T197A</sup> mice (males of 3 months of age) were treated with BAY 11-7082 (5 mg/kg by intraperitoneal injection) or with solvent alone and sacrificed by cervical dislocation after 4 weeks. Results are reported in Supplementary Figure S4A. As shown, treatment with BAY 11-7082 had no apparent effect of the whole weight of both wild type and Cdkn1b<sup>T197A/T197A</sup> mice. In addition, in agreement with the macroscopic data, we observed no microscopic sign of rescue from hyperplasia in Cdkn1b<sup>T197A/T197A</sup> mice treated with BAY 11-7082 (thymus, testis, spleen, small intestine, pituitary gland). We confirmed these findings demonstrating, by immunoblot, that BAY 11-7082 stabilized IkBα, but had no effects on p27 protein levels (Supplementary Figure S4 B-C).

A second set of control experiments was aimed at determining the effects of BZB in Cdkn1b<sup>KO/KO</sup> mice, a mouse strain that presents organomegaly caused by the absence of p27 protein due to a deletion of the Cdkn1b gene (9,11). Since the genetic defect of Cdkn1b<sup>KO/KO</sup> mice is not rescued by proteasome inhibition, we expect that BZB would have no effect on tissue hyperplasia of Cdkn1b<sup>KO/KO</sup> mice if the activity observed in these mice was exclusively dependent on the decrease of p27 turnover.

To this aim we treated control wild type mice (n=3 males and n=3 females) and Cdkn1b<sup>KO/KO</sup> mice (n=3 males and n=3 females) with BZB at the dose 1mg/kg for 4 weeks. Results of this set of experiments are shown in Figure 5.

Macroscopic observation at necroscopy (Figure 5A-E) and histo-pathological analysis of multiple organs explanted from Cdkn1b<sup>WT/WT</sup> mice and Cdkn1b<sup>KO/KO</sup> mice treated with BZB (Fig. 5 F-I) revealed that there was no significant difference in organ size and tissue architecture between Cdkn1b<sup>KO/KO</sup> mice treated with solvent or with BZB. See representative images of thymus, testis, spleen and small intestine of Cdkn1b<sup>KO/KO</sup> mice.

These results strengthen the conclusions that both macroscopic and microscopic phenotypes of the Cdkn1b<sup>T197A/T197A</sup> mice are apparently dependent on p27 levels and not on generic proteasome inhibition.

The turnover of p27<sup>T197A</sup> is regulated by proteasome in murine embryo fibroblasts

To further define the role of T197A in p27 turnover we made use of murine embryonal fibroblasts (MEFs) established from wild type and Cdkn1b<sup>T197A/T197A</sup> mice. First, we determined the rate of p27 turnover in exponentially growing Cdkn1b<sup>T197A/T197A</sup> MEFs that had been incubated with cycloheximide (CHX), an inhibitor of protein synthesis, in presence or absence of BZB. Cdkn1b<sup>WT/WT</sup>...
and Cdkn1bKO/KO MEFs were used as control. As shown in Figure 6A, at difference with p27WT, p27T197A was barely detectable in MEFs at time 0. In the presence of BZB, p27 was markedly increased in Cdkn1bT197A/T197A MEFs and, to a lesser extent, in Cdkn1bWT/WT MEFs. Conversely, p27 protein was completely absent in both control and BZB-treated MEFs derived from Cdkn1bKO/KO MEFs, because of a deletion encompassing exons 1 and 2 of the Cdkn1b gene. Densitometric analysis of p27 levels in the immunoblot of Figure 6A is shown in Figure 6B, and indicated that in wild type MEFs the half-life of p27 is 1.45 h in untreated cells and ≈3.45 h in cells treated with BZB. Conversely, in Cdkn1bT197A/T197A MEFs the half-life of p27 is 1 h in untreated cells and ≈2.45 in cells treated with BZB.

In parallel, we investigated the growth properties of Cdkn1bT197A/T197A MEFs. To this aim we compared cell proliferation of MEFs from Cdkn1bWT/WT (Figure 6C, upper panel), Cdkn1bKO/KO (Figure 6C, middle panel) and Cdkn1bT197A/T197A mice (Figure 6C, lower panel) treated with BZB, BAY-11-7082 or solvent. MEFs from Cdkn1bT197A/T197A and Cdkn1bKO/KO mice duplicated at an accelerated rate (≈1.5 and ≈1.7 fold, respectively) in comparison with Cdkn1bWT/WT MEFs. BZB (1nM and 10nM) but not BAY-11-7082 (1μM and 0.1μM) reduced the proliferation rate of Cdkn1bT197A/T197A MEFs (p<0.001, Two-way Anova) (see Figure 6C, lower panel). Importantly, BZB had no significant effect on the proliferation rate of Cdkn1bKO/KO MEFs (see Figure 6C, middle panel). BZB induced a reduction of 37% at concentration of 1nM (p<0.0001, Two-way Anova) and of 50% at concentration of 10nM (p<0.01, Two-way Anova) in the proliferation rate of Cdkn1bT197A/T197A MEFs compared with Cdkn1bKO/KO MEFs. On the contrary, no significant effect was observed in the proliferation of MEFs from Cdkn1bWT/WT or Cdkn1bKO/KO mice treated with BZB or BAY 11-7082.

Similar results were obtained by analysis of BrdU incorporation. First, MEFs from Cdkn1bT197A/T197A (Figure 6D, lower panel) and Cdkn1bKO/KO (Figure 6D, middle panel) had a significant higher rate of BrdU incorporation compared with Cdkn1bWT/WT MEFs (Figure 6D, upper panel). After 12 hours of serum addition, BrdU incorporation rate was significantly increased in both MEFs from Cdkn1bT197A/T197A and Cdkn1bKO/KO mice compared with Cdkn1bWT/WT MEFs (2.1-fold increase in Cdkn1bT197A/T197A MEFs and 2.9-fold increase in Cdkn1bKO/KO MEFs in comparison with Cdkn1bWT/WT, p<0.005 and p<0.001, respectively).

BZB significantly reduced BrdU incorporation of Cdkn1bT197A/T197A MEFs but not of Cdkn1bKO/KO MEFs. 10nM BZB induced a pronounced decrease in BrdU incorporation of Cdkn1bT197A/T197A MEFs (44% at 12h, 54.1% at 24h and 76.9% at 48h) but not in Cdkn1bKO/KO MEFs (8% at 12h, 29.6% at 24h and 36.9 at 48h) (p<0.05, Student’s t test). Similarly, 1nM BZB induced a decrease in BrdU incorporation of Cdkn1bT197A/T197A MEFs (42.8% at 24h and 57.4% at 48h) but not in Cdkn1bKO/KO MEFs (18.6% at 24h and 11.7 at 48h) (p<0.01, Student’s t test).

On the contrary, BAY 11-7082 induced no significant change in the rate of BrdU incorporation of
Cdkn1b<sup>WT/WT</sup>, Cdkn1b<sup>KO/KO</sup> or Cdkn1b<sup>T197A/T197A</sup> MEFs.
We have generated an ad hoc murine knock-in strain of the Cdkn1b gene (Cdkn1b^{T197A}) that represents an ideal in vivo model to study the effects of inhibiting the abnormally high rate of p27 degradation observed in certain tumors. In this strain, the threonine at position 197 of mouse p27 (T198 in the human) was replaced by an alanine conferring different interesting properties to the protein. First, the presence of alanine at the very C-terminus of p27 makes the protein more prone to proteasome degradation. Second, p27^{T197A} protein can be stabilized and reported to physiological levels by use of proteasome inhibitors (22,23). These properties of Cdkn1b^{T197A/T197A} mice makes this model very attractive to investigate the in vivo effects exerted by p27 restoration to normal levels after proliferative lesions have developed.

Loss of p27 in murine tissues confers a relatively mild phenotype, with increased body size and weight due to multiorgan hyperplasia (9-11). In agreement with these previous studies, we show that the loss of p27 due to increased instability of the protein, leads to increased diffuse hyperplasia. Conversely, the expression of a degradation-resistant version of p27 (Cdkn1b^{T197A}) decreases the development of invasive carcinoma. Overall the conclusions drawn from these studies including the present one suggest that p27 stabilization might be of clinical value (30,31).

Cdkn1b^{T197A} mice, when homozygous, express little p27 in all tissues. This phenomenon is due to the high turnover rate of p27^{T197A} protein. As expected for mice with tissues devoid of p27, Cdkn1b^{T197A} mice present increased size, organomegaly and tissue hyperplasia. Microscopically, Cdkn1b^{T197A/T197A} mice show no alteration in tissue architecture but reveal multiple hyperplastic lesions in most organs, due to increased cell proliferation. These results were further supported by experiments with MEFs established from wild type and Cdkn1b^{T197A/T197A} mice, which indicated that Cdkn1b^{T197A/T197A} MEFs duplicated at an accelerated rate compared with wild type MEFs. Notably, the hyper-proliferative phenotype is rescued by proteasome inhibition, resulting into recovery from organ hyperplasia and drastic reduction of body weight and size.

The phenotype of Cdkn1b^{T197A/T197A} mice is similar to that reported for p27 null mice (Cdkn1b^{KO/KO}) (9,11). However, they present intrinsic differences in the molecular mechanisms that lead to loss of p27 or in the sensitivity to BZB. In the case of Cdkn1b^{KO/KO} the expression of p27 is absent in all tissues because the Cdkn1b gene itself is missing and thus cannot be reactivated by proteasome inhibition. Conversely, in Cdkn1b^{T197A/T197A} mice the lack of p27 is due to a marked increase in the turnover rate of p27^{T197A} protein, which can be stabilized by proteasome inhibition. This difference accounts for the finding that BZB increases p27 half-life and causes a parallel reduction in the proliferation rate in Cdkn1b^{T197A/T197A} MEFs but not in Cdkn1b^{KO/KO} MEFs.

In the cells, the level of p27 protein is regulated by ubiquitin-dependent proteolysis (15,32). P27 turnover is controlled by the activity of the F-Box protein Skp2, which facilitates polyubiquitylation
of p27 by the SCF complex (16,19,33). Indeed the Skp2/p27 interaction represents an attractive target for pharmacological intervention. However, the adoptive re-expression of p27 induces apoptosis in tumour cells (34,35) but not in non-transformed cells that lack it, in which it induces a G0/G1 arrest. The Cdkn1b<sup>T197A</sup> mice generated and characterized here represent a very attractive model to investigate the in vivo effects exerted by p27 restoration to normal levels after hyperplastic lesions have developed. Accordingly, we show that the use of BZB at pharmacological doses increased the levels of p27 protein in the organs of Cdkn1b<sup>T197A/T197A</sup> mice, in parallel with a significant reduction of organ and/or body weight. The effects exerted by BZB in the hyperplastic tissues of Cdkn1b<sup>T197A/T197A</sup> mice are of particular relevance because this compound is currently used in the clinical practice (28,36). The initial rationale for use of BZB in multiple myeloma was that it inhibited proteasome-dependent degradation of IkB, thus limiting NFκB signalling (28). Subsequently, proteasome inhibitors were shown to inhibit the degradation of multiple cellular proteins including p27, p21 and cyclins (29,37). Notably, in agreement with the findings in studies in Multiple Myeloma (36), we did not observe any significant toxicity in mice treated with BZB. Accordingly, BZB is apparently well tolerated in human, since it can be administered in the outpatient setting with low toxicity (38). In the mouse, BZB has been shown to lead to significant reduction of tumour size with minimal toxicity, despite the profound proteasome inhibition observed in normal tissues (39). It appears that actively dividing cells are more sensitive to proteasome inhibition than quiescent or differentiated cells, thus uncovering alterations of cell-cycle checkpoints (28,40,41).

Several findings indicated that the observed reduction in the number and/or degree of hyperplastic lesions induced by BZB was due to reduction of p27 turnover. First, our data indicate that the restoration of p27 levels is necessary for the rescue of the hyper-proliferative phenotype exerted by BZB. In agreement with this concept, BZB induces p27 protein stabilization in the tissues of Cdkn1b<sup>T197A/T197A</sup> mice, where the lack of p27 is due to the intrinsic instability of p27<sup>T197A</sup> protein. However, it fails to reduce hyperplasia in the case of Cdkn1b<sup>KO/KO</sup> mice, because the complete absence of p27 protein is dependent on a genetic defect that cannot be rescued by proteasome inhibitors. Second, BAY 11-7082, a specific inhibitor of NFκB that stabilizes IkB but not p27 (42) has no apparent effect of the whole weight of Cdkn1b<sup>T197A/T197A</sup> mice and fails to rescue hyperplasia, indicating that the anti-hyperplastic effect of BZB in Cdkn1b<sup>T197A/T197A</sup> mice was not mediated by inhibition of NFκB signalling. Third, in agreement with the mouse data, BZB increases p27 half-life and reduces the proliferation in MEFs derived from Cdkn1b<sup>T197A/T197A</sup> but not from Cdkn1b<sup>KO/KO</sup> mice. Fourth, at difference with BZB, BAY 11-7082 had no effect on p27 half-life and on the proliferation rate of Cdkn1b<sup>T197A/T197A</sup> MEFS.

These results are in agreement with a previous study that showed that the antitumoral drug argyrin A exerts its effects by stabilizing p27 through a potent inhibition of the proteasome (43). Argyrin A
influences three different aspects of tumour growth including cell proliferation, apoptosis and angiogenesis, all of which apparently depending on the stabilization of p27. However, although multiple previous works had pointed to an apoptosis-inducing function of p27, our results indicated that BZB had effects on cell proliferation but not on apoptosis. This discrepancy can be explained by the fact that all the studies that showed p27-dependent apoptosis were performed by over-expressing p27 in tumor cells at supra-physiological levels (44-47). Conversely, p27 expression in Cdkn1b<sup>T197A/T197A</sup> mice is regulated by its own promoter and thus occurs at physiological levels. A possible caveat of this study resides in the p27 property to function as assembly factor for cyclin D/cdk4 complexes (48). In this case, by facilitating cyclin D kinase activation, p27 re-expression would promote tumor progression. However, it is difficult to experimentally determine to which extent the assembly function of p27 contributes to tumorigenesis (49), though the phenotypes shown by Cdkn1b<sup>T197A/T197A</sup> and Cdkn1b<sup>KO/KO</sup> mice suggest that this effect may be minimal.

In conclusion, we have generated an ad hoc murine knock-in model of the cell cycle inhibitor p27 to study the effects exerted by restoration of p27 protein in murine tissues that express little or no p27 protein. We show that preventing p27 degradation can be beneficial in the treatment of several benign (and possibly malignant) hyperproliferative disorders. Accordingly, a recent study from Stephens and colleagues (50) demonstrated that Cdkn1b is one of the 18 most significantly mutated genes in luminal A-type breast cancer and identified the specific T198A mutation under study here in one breast cancer patient. Interestingly, the majority of the mutations identified in that study resided in the C-terminal portion of p27, reinforcing the idea that the C-terminus of p27 protein contribute to its tumour suppressor role. Notably one mouse among those under study here was diagnosed with breast cancer at necroscopy before 12 months, underlining the importance to further investigate the role of p27 T198A mutation in human cancer.

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**Figure Legends**

**Figure 1. Characterization of Cdkn1bT197A/T197A mice.** A. Representative growth curves of male and female mice. Graphs show mean ± SD (N=10; p<0.0001, Two-way Anova). B. Weight distribution of small intestine (**) p<0.005, uterus (***)p<0.001, spleen (***)p<0.0005 (N=10, Mann-Whitney test). C. Representative images of small intestine, uterus and spleen of Cdkn1bT197A/T197A or Cdkn1bWT/WT mice.

**Figure 2. T197A replacement of p27 promotes hyperplasia in vivo.** A. Hematoxylin/eosin staining of tissues of Cdkn1bT197A/T197A (magnification x40) or of Cdkn1bWT/WT (in the inset, magnification x10) B. Ki67 staining of tissues from Cdkn1bWT/WT or Cdkn1bT197A/T197A mice of 3 months of age (original magnification x10). C. Quantification of Ki67 staining. Bars represent the mean number of positive nuclei ±SD. (N=3, *p<0.05, **p<0.01; Student’s t test).

**Figure 3. BZB reverts the increase in size and weight of organs of Cdkn1bT197A/T197A mice.** A. Weight distribution of mice (N=10/group). The graph shows mean ± SD. (**) p<0.05, ns=not significant; Two-way Anova). B-D. Weight of organs explanted from Cdkn1bWT/WT or Cdkn1bT197A/T197A mice treated with vehicle (V) or BZB (*p<0.05, **p<0.01, ***p<0.0005; Mann-
Whitney test). E. Representative images of thymuses, uteri and spleens. F. Values of p27/actin ratio as determined by densitometric analysis of immunoblots of the corresponding organs (n=6/group) (*p<0.05, **p<0.005; Mann-Whitney test). G. Immunoblot analysis of p27 in organs explanted from Cdkn1b^{WT/WT} or Cdkn1b^{T197A/T197A} mice treated with V or BZB. Identification number of mice is reported above panels.

**Figure 4. BZB reverts hyperplasia in Cdkn1b^{T197A/T197A} mice.** Hematoxylin/eosin staining of small intestine (A), mammary gland (B), ovary (C), uterus (D), testis (E), thymus (F) and pituitary gland (G) from Cdkn1b^{WT/WT} and Cdkn1b^{T197A/T197A} mice treated with vehicle (V) or BZB. Original magnification, x10.

**Figure 5. BZB does not affect size organ increase and tissue hyperplasia of Cdkn1b^{KO/KO} mice.** A-D. Gross macroscopic analysis of thymus (A), testis (B), spleen (C) and intestine (D) of Cdkn1b^{WT/WT} or Cdkn1b^{KO/KO} mice treated with V or BZB. E. Weight of organs explanted from Cdkn1b^{WT/WT} or Cdkn1b^{KO/KO} mice treated with vehicle (V) or BZB. Bars represent mean ± SD (N=5/group). G-I. Hematoxylin/eosin analysis of pituitary gland (F), small intestine (G), testis (H), spleen (I) of Cdkn1b^{WT/WT} or Cdkn1b^{KO/KO} mice treated with V or BZB. Original magnification x10.

**Figure 6. BZB restores p27 levels in MEFs from Cdkn1b^{T197A/T197A} mice.** A. Immunoblot analysis of p27 half-life in MEFs from Cdkn1b^{WT/WT}, Cdkn1b^{T197A/T197A} or Cdkn1b^{KO/KO} mice, respectively. Cells were treated with 10 μg/mL CHX in presence or absence of BZB (10nM) and collected at the indicated time. B. Densitometric analyses of the blots reported as percentage of remaining protein with respect to the 0h point cells set at 100%. C. Proliferation analysis of MEFs from Cdkn1b^{WT/WT}, Cdkn1b^{KO/KO} or Cdkn1b^{T197A/T197A} mice, in presence or absence of BZB and BAY 11-7082 (BAY), as assessed by Trypan Blue exclusion assay. Data shown in the Figure are means ± SD in triplicate experiments. D. BrdU incorporation assay of MEFs from Cdkn1b^{WT/WT}, Cdkn1b^{KO/KO} or Cdkn1b^{T197A/T197A} mice treated with the indicated doses of BZB or BAY. Graphs show mean ± SD of three independent assays.
Figure 2

(A) Histological images of tissue specimens from different organs: Intestine, Uterus, and Ovary.

(B) Comparison of tissue specimens stained with different markers: Cdkn1b^WT/WT^ vs. Cdkn1b^T197A/T197A^.

(C) Quantitative analysis of Ki67 positive nuclei in different organs: Thymus, Uterus, Intestine, and Testis, showing statistical significance with stars indicating p-values.
Figure 3

A) Body weight (g) over time (weeks) for different genotypes: Cdkn1bWT/WT, Cdkn1bWT/WT + BZB, Cdkn1bT197A/T197A, and Cdkn1bT197A/T197A + BZB. Significant differences are indicated by ** and ns.

B) Thymus weight (mg) for different genotypes: Cdkn1bWT/WT, Cdkn1bWT/WT + BZB, Cdkn1bT197A/T197A, and Cdkn1bT197A/T197A + BZB.

C) Uterus weight (mg) for different genotypes: Cdkn1bWT/WT, Cdkn1bWT/WT + BZB, Cdkn1bT197A/T197A, and Cdkn1bT197A/T197A + BZB.

D) Spleen weight (mg) for different genotypes: Cdkn1bWT/WT, Cdkn1bWT/WT + BZB, Cdkn1bT197A/T197A, and Cdkn1bT197A/T197A + BZB.

E) Images of thymus, uterus, and spleen for different genotypes: Cdkn1bWT/WT, Cdkn1bWT/WT + BZB, Cdkn1bT197A/T197A, and Cdkn1bT197A/T197A + BZB.

F) Graphs of p27/actin ratio for uterus, small intestine, and thymus for different genotypes: Cdkn1bWT/WT, Cdkn1bWT/WT + BZB, Cdkn1bT197A/T197A, and Cdkn1bT197A/T197A + BZB.

G) Western blots for p27 and actin expression in uterus, small intestine, and thymus for different genotypes: Cdkn1bWT/WT, Cdkn1bWT/WT + BZB, Cdkn1bT197A/T197A, and Cdkn1bT197A/T197A + BZB.
Figure 4

Cdkn1b<sup>WT/WT</sup> vs Cdkn1b<sup>T197A/T197A</sup>

A. SMALL INTESTINE

B. MAMMARY GLAND

C. OVARY

D. UTERUS

E. TESTIS

F. THYMUS

G. PITUITARY GLAND
Figure 6

A. Western blot analysis of p27 and Actin levels in Cdkn1bWT/WT, Cdkn1bT197A/T197A, and Cdkn1bKO/KO cell lines treated with NT and BZB.

B. Graph showing the percentage of p27 remaining over time in different treatment conditions.

C. Graphs showing cell number over time for Cdkn1bWT/WT, Cdkn1bT197A/T197A, and Cdkn1bKO/KO cell lines treated with NT, BZB 1nM, BZB 10nM, BAY 0.1μM, and BAY 1μM.

D. Graphs showing BrdU incorporation over time for Cdkn1bWT/WT, Cdkn1bT197A/T197A, and Cdkn1bKO/KO cell lines treated with different concentrations of BZB and BAY.
Molecular Cancer Therapeutics

The T197A knock-in model of Cdkn1b gene to study the effects of p27 restoration in vivo.

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