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Original Research

Effects of the multikinase inhibitors Sorafenib and Regorafenib in PTEN deficient neoplasias

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Abstract The phosphatidylinositol 3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/AKT/mTOR) axis is frequently dysregulated in cancer due to mutations in different nodes of the pathway or constitutive activation of receptor tyrosine kinases. Multikinase inhibitors as sorafenib and regorafenib represent a therapeutic approach for the treatment of these types of tumours. In the present study, we have evaluated the anti-tumoural effects of Sorafenib and Regorafenib on endometrial, prostate and thyroid neoplasias. Both inhibitors reduced cell viability *in vitro* and lead to a disruption of the PI3K/AKT/mTOR pathway. *In vivo*, we have demonstrated that Sorafenib and Regorafenib reduce thyroid hyperplasias induced by the loss of phosphatase and tensin homolog deleted on chromosome 10 (PTEN), although none of the treatments eliminated the disease. Altogether, we present the first study that correlates the response to multikinase inhibitors with a specific mutation. Moreover, this is the first report characterising the response to Regorafenib in thyroid, prostate and endometrial neoplasias.

1. Introduction

The phosphatidylinositol 3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/AKT/mTOR) signalling pathway is a key regulator of many cellular processes including proliferation, cell survival, apoptosis

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and protein synthesis [1]. The canonical PI3K/AKT/mTOR pathway is activated by the binding of growth factors to receptor tyrosine kinases (RTKs). Ligands for RTKs include vascular endothelial growth factor, epidermal growth factor, fibroblast growth factor and other growth factors important for tumour progression and angiogenesis. Binding of those factors to their cognate RTKs leads to phosphorylation and recruitment of PI3K to the plasma membrane. Active PI3K phosphorylates phosphatidylinositol 4,5-biphosphate (PIP_2) to phosphatidylinositol 3,4,5-triphosphate (PIP_3) and subsequently, PIP_3 activates AKT. Phosphorylated AKT dissociates from plasma membrane and acts as a kinase on multiple proteins, thus promoting cell proliferation, survival and cell growth [2]. This signalling cascade is frequently activated in cancer, playing an essential role during the progression of the disease and the development of resistance to treatments [3,4]. In addition to inherent mutations in members of the PI3K/AKT/mTOR pathway, aberrant activation of this cascade can be caused by constitutively activation of RTKs [5]. Therefore, alterations in RTKs signalling play a pivotal role during cancer development and progression.

Negative regulation of the PI3K/AKT/mTOR pathway is mainly accomplished by the action of phosphatase and tensin homolog deleted on chromosome 10 (PTEN), a dual lipid and protein phosphatase that catalyzes dephosphorylation of PIP_3 to PIP_2 [6]. PTEN is one of the most important tumour suppressor genes, and loss-of-function mutations of PTEN have been reported in different cancers including prostate, thyroid, endometrial, breast, colon and haematopoietic malignancies in up to 80% of patients, depending on the type of tumour [4]. The role of PTEN in tumourigenesis has been evidenced by PTEN-knockout mouse models. Mice heterozygous for PTEN ($PTEN^{+/-}$) develop multiple neoplasias [7–9]. Moreover, tissue-specific and inducible biallelic PTEN deletion has been achieved by crossing conditional PTEN floxed mice with different Cre expressing animals [10]. These mouse models are a valuable tool for pre-clinical studies. Since loss of PTEN leads to hyperactivation of the PI3K/AKT/mTOR cascade, multikinase inhibitors represent a therapeutic alternative for PTEN-deficient malignancies.

Sorafenib (Bay 43-9006 or Nexavar) is a multikinase inhibitor initially identified as a Raf-1 inhibitor, although subsequent studies have demonstrated their activity over different kinases including vascular endothelial growth factor receptor, FMS-like tyrosine kinase 3 (FLT-3), c-Kit, Ret and platelet-derived growth factor receptor (PDGFR) [11]. Sorafenib was the first anti-angiogenic multikinase inhibitor to be approved by the US Food and Drug Administration (FDA), and it is currently used for treatment of patients with renal carcinoma, unresectable hepatocellular carcinoma and thyroid cancer [12,13]. Regorafenib (BAY 73-4506 or Stivarga) is an oral multikinase inhibitor structurally related to Sorafenib, leading to a similar but distinct biochemical profile [14]. After successful clinical studies, Regorafenib was approved by the FDA for the treatment of inoperable gastrointestinal stromal tumour [15] and colorectal cancer [16].

In this study, we have assessed the anti-tumoural effects of Sorafenib and Regorafenib on endometrial, prostate and thyroid tumours. We have first evaluated the effects of these multikinase inhibitors on viability and PI3K/AKT/mTOR activation of different cancer cell lines. Next, we used an inducible PTEN-deficient mouse model to characterise the response to the inhibitors on thyroid, prostate and endometrium. Sorafenib and Regorafenib lead to shrinkage of thyroid hyperplasia, but cause more limited or no effects on prostate and endometrial lesions. Finally, we assessed the response to continuous treatment with both multikinase inhibitors. To date, this is the first pre-clinical study evaluating the response to either Sorafenib or Regorafenib in thyroid hyperplasias and prostate or endometrial neoplasias induced by PTEN loss. Collectively, we show that both drugs represent a useful therapeutic approach for PTEN-deficient thyroid lesions, while Sorafenib also reduce prostate neoplasias. Despite both treatments reduced cell proliferation, none of them eliminated the disease.

2. Materials and methods

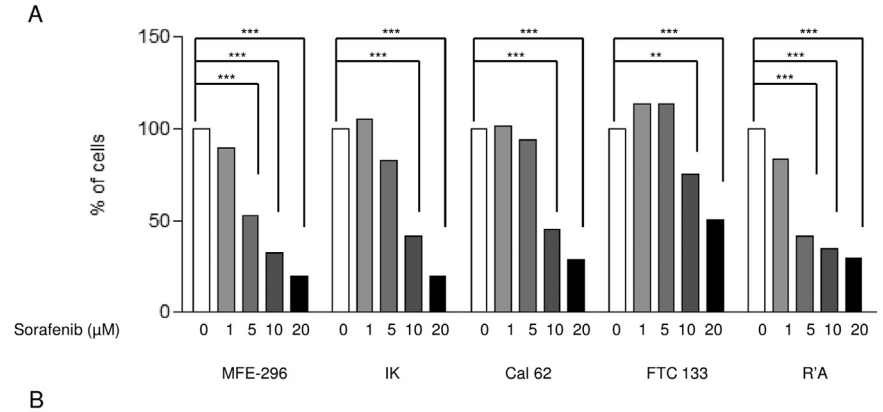
2.1. Mice

Mice were housed and maintained in a barrier facility, and pathogen-free procedures were used in all mouse rooms. Up to 15 mice were housed together in each cage and kept in a 12-h light/dark cycle at 22 °C with *ad libitum* access to autoclaved food and water.

$Cre{:}ER^{T+/-}$ $PTEN^{fl/fl}$ mice were generated and handled as previously described [10]. Briefly, 3 weeks after birth, animals were weaned and tails were cut in order to genotype them. Isolation and polymerase chain reaction analysis of tail genomic DNA were performed as previously described [17,18].

During all the studies, humane end-points were used. Mice were monitored every day and euthanised when they showed any clinical signs of disease. Animals were anaesthetised with isofluorane and sacrificed by cervical dislocation. Organs were collected and further processed for the different studies described below.

All procedures performed in this study followed the National Institute of Health Guide for the Care and Use of Laboratory Animals and were compliant with the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100
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Sorafenib (μM)
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MFE-296
IK
Cal 62
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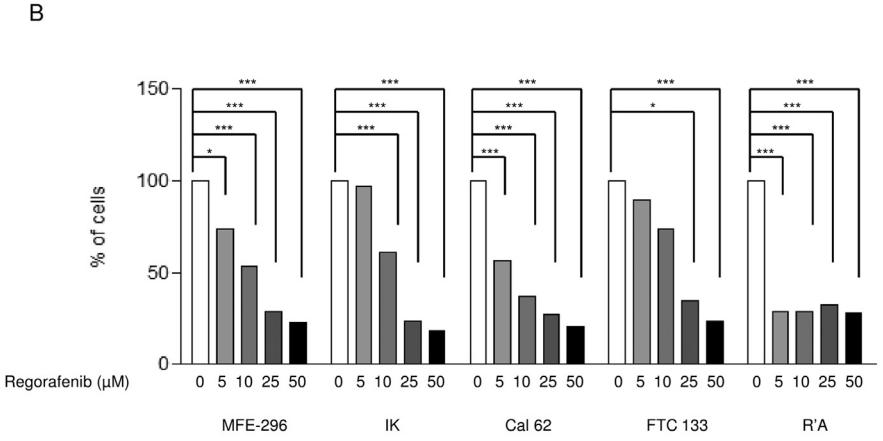
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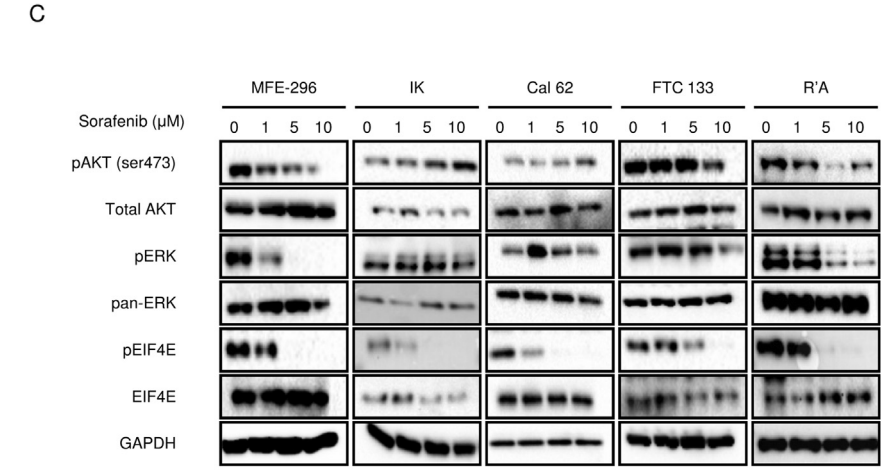
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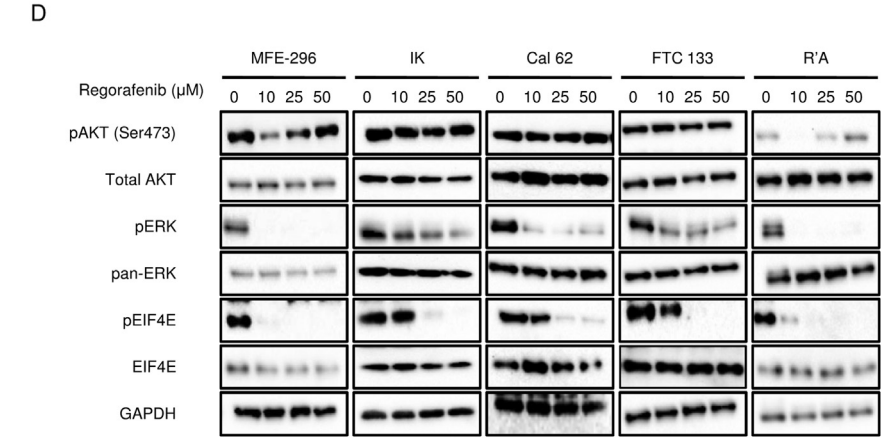
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Total AKT
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pEIF4E
EIF4E
GAPDH

Committee on Ethics of Animal Experiments of Universitat de Lleida/IRB Lleida.

2.2. Pharmacological treatments

To induce PTEN deletion, adult mice (4–5 weeks old) were given a single intraperitoneal injection of 0.5 mg of tamoxifen (T5648 Sigma–Aldrich) emulsion (30–35 μg per mg body weight).

Sorafenib (BAY 54-9085, Bayer) was dissolved in a 50% Kolliphor® EL (C5135, Sigma–Aldrich) and 50% absolute ethanol (Scharlau) mixture to a concentration of 60 mg/mL. Mice were given a single daily dose of 60 mg/kg of Sorafenib solution by oral gavage starting 3 weeks after tamoxifen injection.

Regorafenib (BAY 73-4506, Bayer) was dissolved in a 1:1:2 solution of 1,2-propylenglycol (Fluka 82281):PEG 400 (Fluka 81172):Pluronic F68 (VRW) to obtain a stock solution of 200 μg/mL. Working solution was freshly prepared everyday by adding 20% of sterile water. Mice received a single daily dose of 60 mg/kg of Regorafenib solution by oral gavage starting 3 weeks after tamoxifen injection.

Both drugs were a generous gift from BayerHealthcare Pharmaceuticals.

2.3. Cell culture and in vitro treatments

The Ishikawa 3-H-12 (IK) and MFE-296 endometrial cancer cell lines were purchased from the American Type Culture Collection. Cal 62 and FTC 133 thyroid cells were a gift from Dr. Santisteban (Instituto de Investigaciones Biomédicas Alberto Sols, Madrid). R33327-5'A (R'A) cells were donated by Dr. Garí (IRB Lleida). All cell lines were grown in Dulbecco's modified Eagle medium (Sigma–Aldrich) supplemented with 10% foetal bovine serum (Invitrogen), 1 mmol/L HEPES (Sigma–Aldrich), 1 mmol/L sodium pyruvate (Sigma–Aldrich), 2 mmol/L L-glutamine (Sigma–Aldrich), and 1% of penicillin/streptomycin (Sigma–Aldrich) at 37 °C with saturating humidity and 5% CO_2. All experiments were conducted with low passage cells from recently resuscitated frozen stocks.

A 50-mM stock of Sorafenib or 10 mM of Regorafenib was prepared in dimethyl sulphoxide (DMSO) and stored as single use aliquots at −80 or −20 °C, respectively, until further use. For each experiment, drug dilutions were prepared in fresh growth medium.

2.4. Viability assays

Cell viability was evaluated as general mitochondrial activity of the cells by assaying reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan. Briefly, 7.5×10^3 cells were plated on M96-well plates and incubated for 30 min with 0.5 mg/mL of MTT reagent after the indicated treatments. Next, growth medium was removed and replaced with DMSO to dissolve the formazan crystals produced by the mitochondrial succinate dehydrogenase of the living cells. Finally, absorbance was measured using a spectrophotometer (Bio-Rad) at a dual wavelength of 595 and 620 nm. Cell viability was expressed as the percentage of absorbance obtained from living cells surviving after drug treatment relative to untreated cells.

2.5. Western blot analysis

Total protein extraction was performed from frozen thyroid, prostate and uteri. Briefly, tissues were disaggregated with glass beads (Sigma–Aldrich) in a 5M urea solution using a disruptor FastPrep FP120 (BIO101) for 45 s at high power. Next, samples were boiled, re-dissolved in lysis buffer (2% sodium dodecyl sulphate, 125 mmol/L Tris-HCl, pH 6.8) and centrifuged at 7000 rpm for 5 min. Finally, supernatants were collected. For cell cultures, total protein was extracted by using the same lysis buffer and protein concentrations were determined with a protein assay kit (Bio-Rad). Equal amounts of proteins were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to PVDF membranes (Millipore). Non-specific binding was blocked by incubation with TBST (20 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, and 0.1% Tween 20) plus 5% non-fat milk. Membranes were incubated with the primary antibody overnight at 4 °C followed by a 1-h incubation with secondary antibody anti-mouse or anti-rabbit (Jackson). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin were used as loading control. Signal was detected with ECL Advance (Amersham-Pharmacia). The antibodies used were against phospho-AKT Ser473 (Cell Signalling #4060), total AKT (Santa Cruz, sc-1618), phospho-ERK 1/2 (Cell Signalling #9101), total ERK

Fig. 1. Sorafenib and Regorafenib reduce cell viability in endometrial, thyroid and prostate cancer cell lines. (A) MFE-296, IK, Cal-62, FTC 133 and R'A cells were treated for 48 h with increasing doses of Sorafenib or (B) Regorafenib and cell viability was evaluated by MTT. Results are expressed as percentage of survival over control values. (C) Cell lines were treated with Sorafenib or (D) Regorafenib for 16 h and expression of different members of the PI3K/AKT/mTOR pathway was evaluated by Western blot. Abbreviations: IK, Ishikawa 3-H-12; R'A, R33327-5'A; PI3K/AKT/mTOR, phosphatidylinositol 3-kinase/protein kinase B/mammalian target of rapamycin.

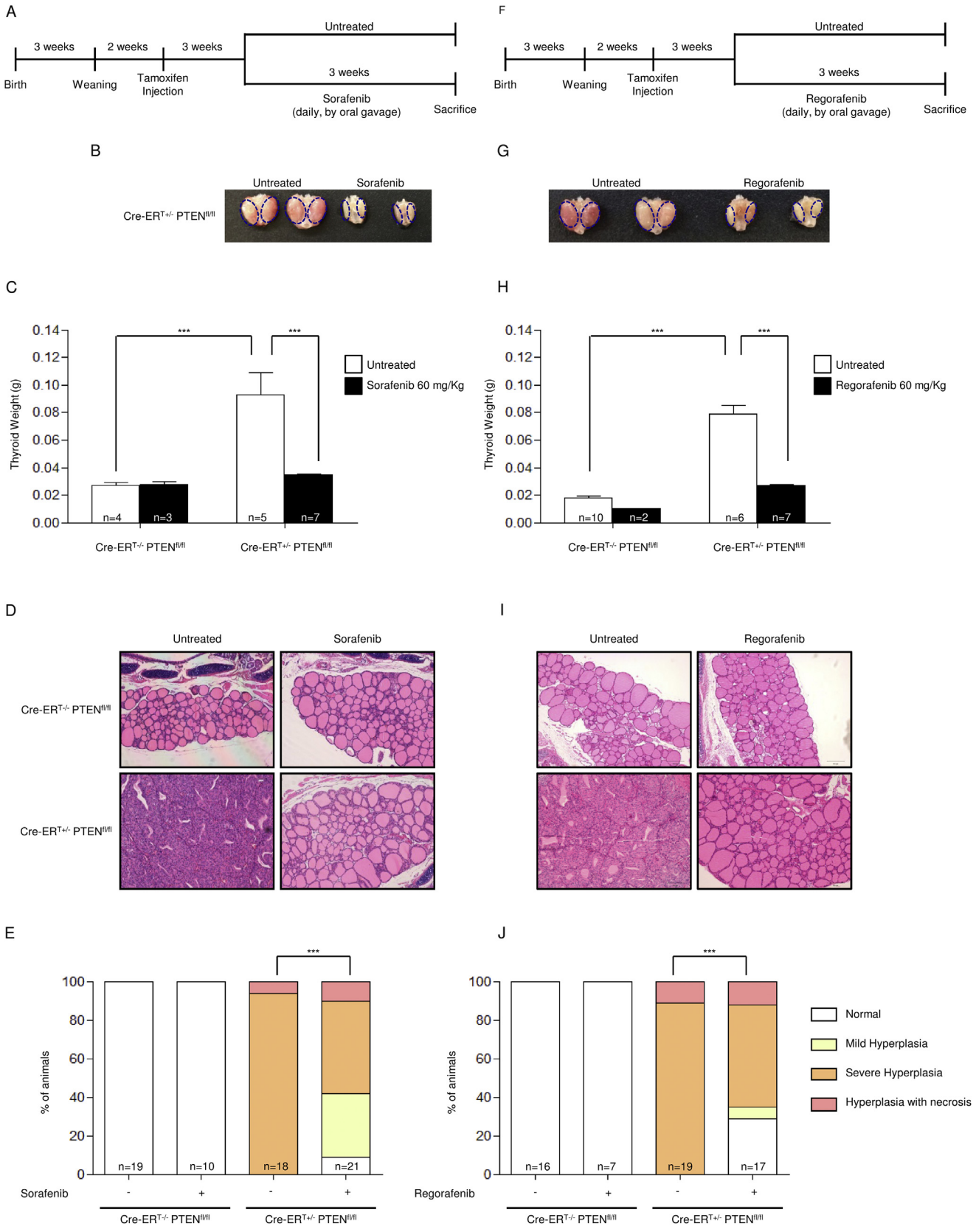


Fig. 2. Multikinase inhibitors reduce thyroid hyperplasia in PTEN-deficient mice. (A) Schematic representation of the protocol used for Sorafenib administration. Briefly, mice were given a single daily dose of 60 mg/kg of the drug for 21 consecutive days, starting 3 weeks after PTEN deletion. (B and C) Macroscopic images and weight of thyroids from Sorafenib-treated mice. (D and E) Representative images of HE staining (×10) and evaluation of lesions of thyroid from Sorafenib-treated animals. (F) Schematic representation of the protocol

(BD610623), phospho-eIF4E (Upstate #07-823), total eIF4E (Santa Cruz, sc-9776), GAPDH (Abcam #8245) and actin (Santa Cruz, sc-1616).

2.6. Histopathology and immunohistochemical analysis

Animals were euthanised and organs were collected, formalin fixed overnight at 4 °C and embedded in paraffin for histological examination. Paraffin blocks were sectioned at a thickness of 3 μm and dried for 1 h at 65 °C before pre-treatment procedure of deparaffinisation, rehydration and epitope retrieval in the Pre-Treatment Module, PT-LINK (DAKO) at 95 °C for 20 min in 50× Tris/EDTA buffer, pH 9. Before staining the sections, endogenous peroxidase was blocked. The antibodies used were against cyclinD1 (Dako, M3642 ready to use) and PTEN (Dako, Denmark, clone 6H2.1, 1/100 dilution). After incubation, the reaction was visualised with the EnVision FLEX Detection Kit (DAKO, Denmark) using diaminobenzidine chromogen as a substrate. Sections were counterstained with haematoxylin. Appropriate negative controls were also tested. Representative images were taken with a Leica DMD 108 microscope.

Haematoxylin and eosin (HE)–stained samples were histologically reviewed and evaluated by two pathologists, following uniform pre-established criteria. Representative images were taken with a Leica DMD 108 microscope.

2.7. Statistical analyses

All the experiments were performed and repeated at least three times. *N* indicates the number of mice. Statistical analyses were performed using Prism 6.0 software (GraphPad). Statistical significance was evaluated using analysis of variance followed by Bonferroni's test for multiple comparisons. P value ≤ 0.05 was considered statistically significant.

3. Results

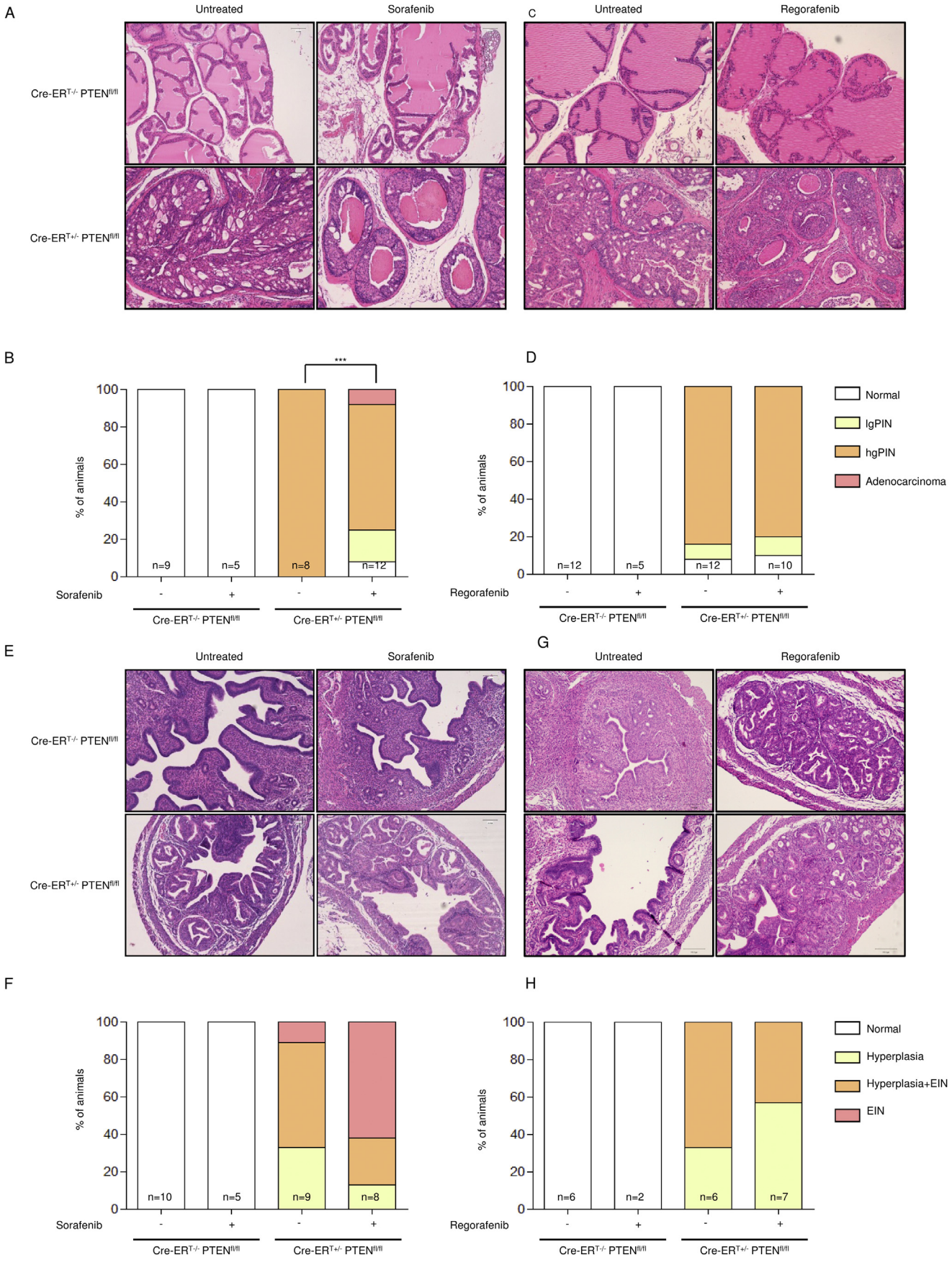
3.1. Sorafenib and Regorafenib treatment reduce in vitro viability on endometrial, thyroid and prostate cell lines and cause a disruption of PI3K/AKT/mTOR axis

Previous studies from our laboratory have shown that endometrial cell lines are sensitive to anti-tumoural effects of Sorafenib treatment [19]. For this reason, we decided to compare the *in vitro* response of the multikinase inhibitors Sorafenib and Regorafenib on endometrial, thyroid and prostate cells lines. For this purpose, we used two endometrial cancer cell lines, MFE-296 (PTEN mutated) and Ishikawa (PTEN null), two thyroid cells, Cal 62 (PTEN wild type) and FTC 133 (PTEN wild type), and one more from prostate origin, R'A (PTEN wild type). All five cell lines were treated with increasing doses of each inhibitor for 48 h and viability was evaluated by a MTT cytotoxicity assay. All the cell lines evaluated showed a dose-dependent sensitivity to Sorafenib (Fig. 1A) and Regorafenib (Fig. 1B). Next, we sought to characterise the molecular mechanism behind Sorafenib and Regorafenib treatments. Among all signalling pathways activated by RTKs, the PI3K/AKT/mTOR pathway plays a pivotal role in driving their cellular responses; hence, we hypothesised that both multikinase inhibitors could be disrupting this axis to reduce cell viability. All five cell lines were treated with increasing doses of Sorafenib or Regorafenib for 16 h and protein expression was evaluated by Western blot. The different cell lines displayed different responses depending on both the dose and the inhibitor used (Fig. 1C,D), but all of them showed a dramatic decrease of the phosphorylation of the translation factor eIF4E. These results suggest that Sorafenib and Regorafenib cause a disruption of the PI3K/AKT/mTOR pathway at different levels and affecting different components, but converging towards a common downstream effector.

3.2. Sorafenib and Regorafenib reduce PTEN-induced thyroid hyperplasia in vivo

Having demonstrated that reduction of cell viability caused by Sorafenib and Regorafenib *in vitro* correlated with inhibitory effect on the PI3K/AKT/mTOR axis, we decided to evaluate their therapeutic potential *in vivo*. For this purpose, we used a tamoxifen-inducible PTEN knockout mouse model (Cre-ER$^{T+/-}$ PTEN$^{fl/fl}$), previously described by our laboratory [10]. Interestingly, administration of a single dose of Tamoxifen results in PTEN deletion both *in vitro* and *in vivo* (Supplementary Fig. 1A,B). These mice develop thyroid hyperplasia, prostate and endometrial neoplasias due to PTEN loss and the consequent hyperactivation of the PI3K/AKT/mTOR pathway, suggesting that Sorafenib and Regorafenib could have a beneficial effect on the disease. Briefly, mice received a single dose of tamoxifen to induce PTEN deletion 5 weeks after birth, and 3 weeks

used for Regorafenib administration. The same administration protocol was used for Regorafenib and Sorafenib. (G and H) Macroscopic images and weight of thyroids from Regorafenib-treated mice. (I and J) Representative images of HE staining (×10) and evaluation of lesions of thyroid from Regorafenib-treated animals. Both Regorafenib and Sorafenib treatments reduced thyroid weight and had beneficial effect on thyroid hyperplasias. Abbreviations: PTEN, phosphatase and tensin homolog deleted on chromosome 10; HE, haematoxylin and eosin.


A
Untreated
Sorafenib
C
Untreated
Regorafenib
Cre-ER$^{T-/-}$ PTEN$^{fl/fl}$
Cre-ER$^{T+/-}$ PTEN$^{fl/fl}$
B
***
% of animals
100
80
60
40
20
0
n=9
n=5
n=8
n=12
Sorafenib
-
+
-
+
Cre-ER$^{T-/-}$ PTEN$^{fl/fl}$
Cre-ER$^{T+/-}$ PTEN$^{fl/fl}$
D
% of animals
n=12
n=5
n=12
n=10
Regorafenib
Normal
lgPIN
hgPIN
Adenocarcinoma
E
Untreated
Sorafenib
G
Untreated
Regorafenib
F
% of animals
n=10
n=5
n=9
n=8
Sorafenib
H
% of animals
n=6
n=2
n=6
n=7
Regorafenib
Normal
Hyperplasia
Hyperplasia+EIN
EIN

later, we started the treatment with the corresponding inhibitor, Sorafenib (Fig. 2A) or Regorafenib (Fig. 2F). Sorafenib and Regorafenib were given daily by oral gavage for 21 consecutive days, and then the animals were sacrificed. Macroscopic analysis revealed a dramatic reduction in thyroid size and weight from Cre-ER$^{T+/-}$ PTEN$^{fl/fl}$ mice treated with either Sorafenib (Fig. 2B,C) or Regorafenib (Fig. 2G,H). Moreover, histopathological evaluation confirmed that the percent of animals showing thyroid hyperplasia was reduced in glands from mice receiving Sorafenib (Fig. 2D,E) or Regorafenib (Fig. 2I,J).

3.3. Sorafenib and Regorafenib effects on PTEN-deficient prostate and endometrial neoplasias

As Cre-ER$^{T+/-}$ PTEN$^{fl/fl}$ mice also develop high-grade prostatic intraepithelial neoplasias (hgPIN) and endometrial intraepithelial neoplasias, we also evaluated the impact of Sorafenib and Regorafenib treatments on these lesions. Both drugs led to a reduction of prostate size, but they caused different effects on the neoplasias. Sorafenib-treated mice displayed prostate lesions of lesser degree than the control group, presenting low-grade prostatic intraepithelial neoplasias, albeit only one of them conserved a normal histology (Fig. 3A,B). On the contrary, no effects were observed on prostates from Regorafenib-treated animals (Fig. 3C,D).

Finally, we analysed the effects of both inhibitors on uterine tumourigenesis. As shown in Fig. 3, neither Sorafenib (Fig. 3E,F) nor Regorafenib (Fig. 3G,H) reduced PTEN-deficient endometrial tumours. Collectively, our data suggest that Sorafenib and Regorafenib have a different impact in different tissues, even when the lesions are induced by a common mutation. Moreover, albeit both inhibitors share most of their molecular targets, their effect on endometrial and prostate lesions is not the same.

3.4. Evaluation of the PI3K/Akt pathway after Sorafenib or Regorafenib treatment of PTEN-deficient lesions

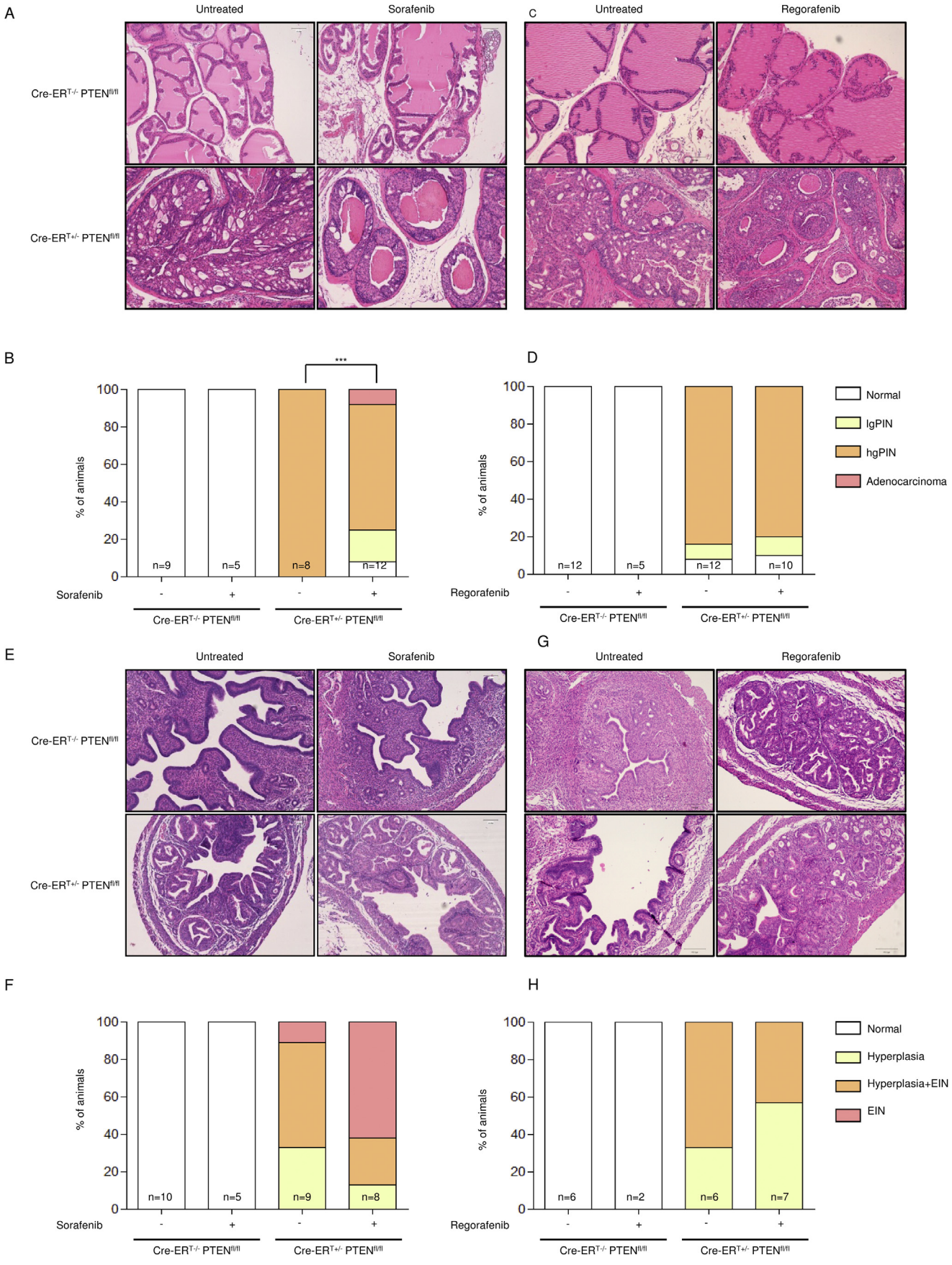
In order to elucidate the molecular mechanism explaining Sorafenib and Regorafenib effects *in vivo* and the differences of their anti-tumoural activity on different tissues, we analysed by Western blot and immunohistochemistry the expression of several components of the PI3K/AKT/mTOR axis in tissues from control or multikinase inhibitor-treated mice. As depicted in Fig. 4A, Sorafenib induced a disruption of the signalling pathway in thyroid and prostate, where phosphorylation of AKT, ERK and eIF4E was diminished, but not in the endometrium. Moreover, cyclin D1 expression was reduced in both tissues, indicating a decrease in cell proliferation (Fig. 4B,C). According to the histopathological analysis, no differences were observed in uteri from control and treated mice (Fig. 4A,D), suggesting a correlation between inhibition of this pathway and the therapeutic effect of Sorafenib. On the other hand, we did not observed downregulation of PI3K/AKT/mTOR pathway with Regorafenib treatment (Fig. 4E), although cyclin D1 expression was lower in thyroids (Fig. 4F) but not in prostate (Fig. 4G) and uteri (Fig. 4H) from treated mice. Altogether, our data indicate that Sorafenib and Regorafenib act throughout different molecular mechanisms, but both of them reduce cell proliferation in PTEN-deficient thyroid hyperplasias.

3.5. Effects of Sorafenib and Regorafenib chronic treatment on thyroid hyperplasia

Having assessed the short-term effects of Sorafenib and Regorafenib, we asked whether an extension of the treatment period could completely eliminate thyroid hyperplasia and prostate neoplasias. Since we did not observe any effect on endometrial tumours after 21 d of treatment, we excluded females from this second study. The treatment scheme for Sorafenib is showed in Fig. 5A; briefly, mice were given a daily dose of Sorafenib starting 3 weeks after tamoxifen injection until they required euthanasia. Additionally, we set up another group that was treated only for 21 days and then kept until they were sacrificed due to illness. The purpose of this last group was to check if short-term treatment was enough for eliminating the disease.

Longer periods of Sorafenib administration practically doubled mice survival from 6–13 weeks after tamoxifen injection (Fig. 5B). Strikingly, a similar effect was observed with the short-term treatment, suggesting that the inhibitor can preserve its anti-tumoural activity even after its administration ceases. As shown in Fig. 5C, thyroids from mice that received the chronic treatment showed similar size to thyroid from control Cre-ER$^{T-/-}$ PTEN$^{fl/fl}$ animals. However, the glands from mice that had received Sorafenib only during 3

Fig. 3. Sorafenib and Regorafenib effects on PTEN-deficient prostate and endometrial neoplasias. (A and B) Representative images of HE staining (×10) and evaluation of prostate histology from Sorafenib-treated or (C and D) Regorafenib-treated animals. Sorafenib treatment improved the extent of the prostatic lesions but Regorafenib showed no effect on prostate neoplasias. (E and F) Representative images of HE staining (×10) and evaluation of endometrial histology from Sorafenib-treated or (G and H) Regorafenib-treated animals. None of the inhibitors showed beneficial effect on the endometrium. lgPIN and hgPIN mean low- or high-grade prostatic intraepithelial neoplasia, respectively. Abbreviations: PTEN, phosphatase and tensin homolog deleted on chromosome 10; HE, haematoxylin and eosin; EIN: endometrial intraepithelial neoplasia.


A
Untreated
Sorafenib
C
Untreated
Regorafenib
Cre-ER$^{T-/-}$ PTEN$^{fl/fl}$
Cre-ER$^{T+/-}$ PTEN$^{fl/fl}$
B
***
% of animals
100
80
60
40
20
0
n=9
n=5
n=8
n=12
Sorafenib
-
+
-
+
Cre-ER$^{T-/-}$ PTEN$^{fl/fl}$
Cre-ER$^{T+/-}$ PTEN$^{fl/fl}$
D
% of animals
n=12
n=5
n=12
n=10
Regorafenib
Normal
lgPIN
hgPIN
Adenocarcinoma
E
Untreated
Sorafenib
G
Untreated
Regorafenib
F
% of animals
n=10
n=5
n=9
n=8
Sorafenib
H
% of animals
n=6
n=2
n=6
n=7
Regorafenib
Normal
Hyperplasia
Hyperplasia+EIN
EIN

weeks were dramatically enlarged, similarly to the untreated group, indicating that short-term treatment was not enough to eliminate malignant cells and completely abrogate the disease. Surprisingly, despite the differences on thyroid size, all three groups of PTEN-deficient mice displayed severe hyperplasia of the gland (Fig. 5D and E), thus demonstrating that Sorafenib treatment slows down the progression but does not cure PTEN-induced thyroid hyperplasia.

Next, we followed a similar approach with Regorafenib. The treatment scheme for Regorafenib is showed in Fig. 5F; briefly, mice were given a daily dose of the drug starting 3 weeks after tamoxifen injection until they required euthanasia. In in this case, the drug showed an important toxicity and the effects of an extended treatment could not be evaluated. Short-term treatment did not increase survival comparing to the untreated group (Fig. 5G). Moreover, no differences were observed regarding thyroid size (Fig. 5H) or histology (Fig. 5I,J) between treated and untreated mice. To overcome the toxicity of Regorafenib, we designed a second experiment in which the animals received a daily dose of the drug for 5 days followed by 9 days of rest before starting the next cycle of treatment (Supplementary Fig. 2A). Interestingly, this approach increased mice survival up to 70 days (Supplementary Fig. 2B), hence confirming a reduction of the toxicity. Nonetheless, histological analysis of the thyroids (Supplementary Fig. 2C,D) revealed no differences due to Regorafenib treatment. Collectively, these results indicate that, albeit Regorafenib slows down the progression of thyroid hyperplasia induced by PTEN loss, it does not eliminate malignant cells completely and the disease progresses.

3.6. Effects of Sorafenib and Regorafenib chronic treatment on prostate neoplasias

Finally, we also characterised the effects of chronic administration of Sorafenib and Regorafenib on PTEN-deficient prostate neoplasias. Surprisingly, Sorafenib led to an improvement of prostate lesions either with long- or short-term treatments. Although the size of the gland was higher than in the control group (Cre-ER$^{T-/-}$ PTEN$^{fl/fl}$) (Fig. 6A), the histological examination revealed a significant reduction of hgPIN due to the treatment (Fig. 6B,C). On the other hand, and equivalent to the observations made in thyroid, Regorafenib treatment did not reduce neither prostate size (Fig. 6D) nor prostate neoplasias and histopathological analysis revealed that all PTEN-deficient mice display hgPIN, with independence of the treatment received (Fig. 6E and F and Supplementary Fig. 2E,F).

4. Discussion

In the present study, we have evaluated the antitumoural effects of the multikinase inhibitors Sorafenib and Regorafenib on thyroid hyperplasia, endometrial and prostate neoplasias. The PI3K/AKT/mTOR pathway is frequently hyperactivated in different tumours, and both the restoration of any of the nodes of the signalling pathway as well as inhibition of RTKs result in increased vulnerability of the tumour to different treatments [20]. RTKs inhibitors (iRTKs) generally cause a suppressive effect on the PI3K/AKT/mTOR axis. However, given that several RTKs are frequently altered simultaneously, tumours become resistant to monotherapy. Multikinase inhibitors as Sorafenib and Regorafenib offer an alternative to overcome monotherapy resistance by inhibiting more than one target with only one compound.

We have observed that, *in vitro*, both Sorafenib and Regorafenib reduce viability of endometrial, thyroid and prostate cancer cell lines. However, we did not observe the same effects *in vivo*. It is important to note that *in vitro*, the drugs can block their targets directly, without any barriers that impede the drugs to reach the tumoural cells. Conversely, when administered *in vivo*, they have to be distributed throughout the body before arriving to their targets. This implies that for some tissues, the bioavailability, and therefore the therapeutic effect, can be altered and dramatically reduced. Moreover, the stroma can modify the tumour microenvironment by producing cytokines and other factors that regulate the tumourigenic process, promoting survival and remodelling the niche [21]. Although all the lesions evaluated in this study were caused by PTEN deletion, it must be considered that extrinsic factors from the

Fig. 4. Characterisation of the PI3K/AKT/mTOR pathway on PTEN-deficient tissues after Sorafenib or Regorafenib treatment. (A) Evaluation of PI3K/AKT/mTOR pathway by Western blot in thyroid, prostate and uteri from Sorafenib-treated mice. Sorafenib disrupted the PI3K/AKT/mTOR signalling axis, by diminishing the levels of p-AKT, p-ERK and p-eIF4E, in thyroid and prostate. (B–D) Representative images (×10) of cyclin D1 immunostaining in thyroid, prostate and uteri from control and Sorafenib-treated mice. Sorafenib treatment induced a reduction of CycD1 expression in thyroid and prostate. (E) Evaluation of PI3K/AKT/mTOR pathway by Western blot in thyroid, prostate and uteri from Regorafenib-treated mice. (F and H) Representative images (×10) of cyclin D1 immunostaining in thyroid, prostate and uteri from control and Regorafenib-treated mice. Regorafenib treatment induced a reduction of CycD1 expression in thyroid but no other effect was observed on the PI3K/AKT/mTOR signalling pathway. Abbreviations: PI3K/AKT/mTOR, phosphatidylinositol 3-kinase/protein kinase B/mammalian target of rapamycin; PTEN, phosphatase and tensin homolog deleted on chromosome 10.

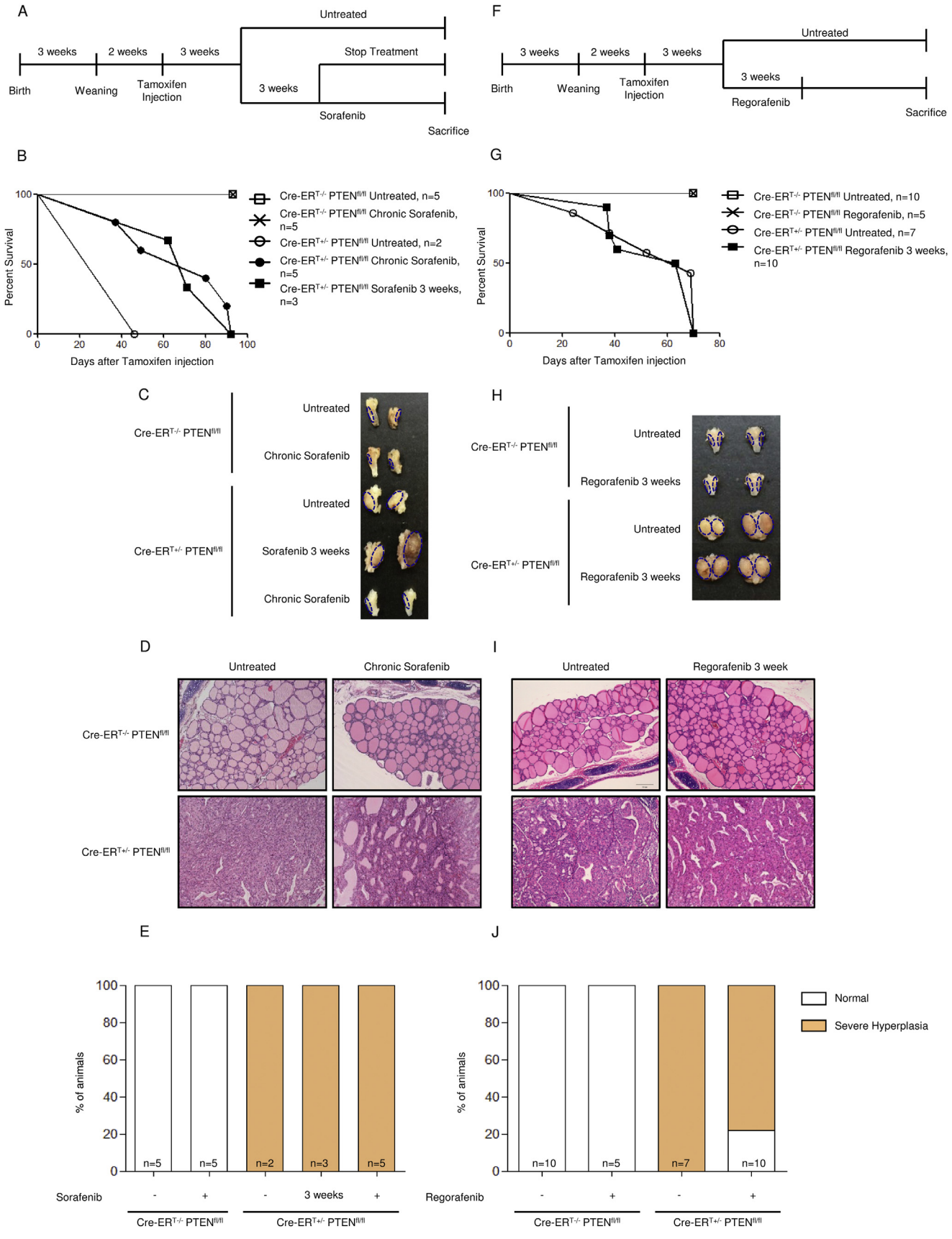


Fig. 5. Effects of chronic treatment with multikinase inhibitors in PTEN-deficient thyroid hyperplasias. (A) Schematic representation of the protocol used for Sorafenib and (B) Kaplan-Meier survival curve. (C) Macroscopic images of thyroids from Sorafenib-treated mice. (D and E) Representative images of HE staining (×10) and evaluation of lesions of thyroid from Sorafenib-treated animals. Sorafenib

tumoural niche may modulate the therapeutic effect and provide an explanation for the different results observed with Sorafenib and Regorafenib among the tumoural types.

Recent studies have shown that inhibition of AKT is required for Sorafenib-induced cell death *in vitro*. Thus, overexpression of AKT reduces the death caused by the drug, whereas co-treatment with the PI3K inhibitor LY294002 increases its cytotoxic effects [22]. Consistent with these findings, we have observed that the cell lines in which Sorafenib decreases AKT phosphorylation are more sensitive to the treatment. Moreover, these results were reproduced *in vivo* in some tissues, as both thyroid and prostate, but not the uterus, showed a disruption of the pathway, correlating with the therapeutic effect. On the contrary, we did not observe such correlation between Regorafenib-induced cell death and inhibition of the PI3K/AKT/mTOR pathway *in vitro* or *in vivo*. Although the treatment induced a disruption of the signalling cascade in all the cell lines tested *in vitro*, the response *in vivo* differed from these results. No differences on PI3K/AKT/mTOR activation were observed among the responsive thyroid hyperplasias and the unresponsive prostate or endometrial neoplasias. Previous research have shown that inhibition of PI3K cooperates with Regorafenib to induce cell death in breast, lung, colorectal, kidney and brain cancer cell cultures [23]. However, most of those evidences have not been validated *in vivo*.

Our results with Sorafenib are in agreement with previous clinical evidence obtained with this inhibitor. Successful results have been reported for thyroid cancer, leading to the approval of the drug for the treatment of refractory thyroid carcinoma in 2013 [13]. Interestingly, Sorafenib represents a new alternative for overcoming resistance of chemotherapy-failure castration-resistant prostate cancer [24]. A recent early-phase clinical trial has shown that the combination of Sorafenib with standard chemotherapy is safe and could overcome resistance to treatment in such patients. Nonetheless, further research with phase II/III is needed to corroborate these observations. The efficacy of Sorafenib has also been determined in patients with endometrial cancer in a phase II trial [25]. Similar to our results, Sorafenib had minimal activity in such patients and predictive factors for potential benefit are needed.

Hyperactivation of the PI3K/AKT/mTOR signalling pathway is associated to resistance to treatment with iRTKs, being responsible for more than 20% of therapy failures [26]. Nonetheless, the role of p-AKT as a possible marker for evaluating the response to these treatments is not fully understood. The identification of biomarkers to predict sensitivity or resistance to therapies is a prior line of research in the field. These types of studies will allow physicians to direct patients to the most convenient treatment, with important consequences regarding both medical and economical aspects. Here, we present the first study where both Sorafenib and Regorafenib are evaluated on neoplasias induced by a single mutation, hence relating their therapeutic potential with a concrete molecular alteration. According to our results, Sorafenib is a promising drug for the treatment of PTEN-induced prostate neoplasias, both inhibitors are useful for treating thyroid hyperplasia and useless for endometrial tumours. It is worth mentioning that all those observations refer to monotherapy treatment and we cannot rule out the possibility that combination of Sorafenib and Regorafenib with other drugs can be more effective for PTEN-induced malignancies.

At the molecular level, the mechanism by which iRTKs induce cellular death is not fully described. However, a common feature observed in various tumour types *in vitro* is the reduction in the phosphorylation of the translation factor eIF4E. In our case, this has been a common feature to both inhibitors in all cell lines *in vitro*. *In vivo*, we only observed such correlation with Sorafenib, suggesting that eIF4E acts as a point of convergence between different signalling axis, thus initiating the effector activity of the drug. On the other hand, no correlation was found between Regorafenib treatment and eIF4E phosphorylation *in vivo*. These results suggest that other pathways besides the PI3K/AKT/mTOR cascade are activated due to Regorafenib treatment. The description and knowledge of these pathways needs further research.

In conclusion, multikinase inhibitors are promising drugs for cancer therapy. Here, we report the first preclinical study evaluating the therapeutic potential of Regorafenib for thyroid hyperplasia, prostate and endometrial neoplasias. Moreover, we compared its effects to Sorafenib treatment and characterised the impact on the PI3K/AKT/mTOR pathway both *in vitro* and *in vivo*. To date, this is the first study where both inhibitors are compared *in vivo* specifically for the treatment of PTEN-induced neoplasias. These results corroborate Sorafenib and Regorafenib as promising candidates for some PTEN-deficient tumours, although

chronic treatment doubled mice survival although it did not reduce thyroid hyperplasia. (F) Schematic representation of the protocol used for Regorafenib and (G) Kaplan-Meier survival curve. (H) Macroscopic images of thyroids from Regorafenib-treated mice. (I and J) Representative images of HE staining (×10) and evaluation of lesions of thyroid from Regorafenib-treated animals. No effect of Regorafenib was observed on survival and thyroid weight and histology. Abbreviations: HE, haematoxylin and eosin; PTEN, phosphatase and tensin homolog deleted on chromosome 10.

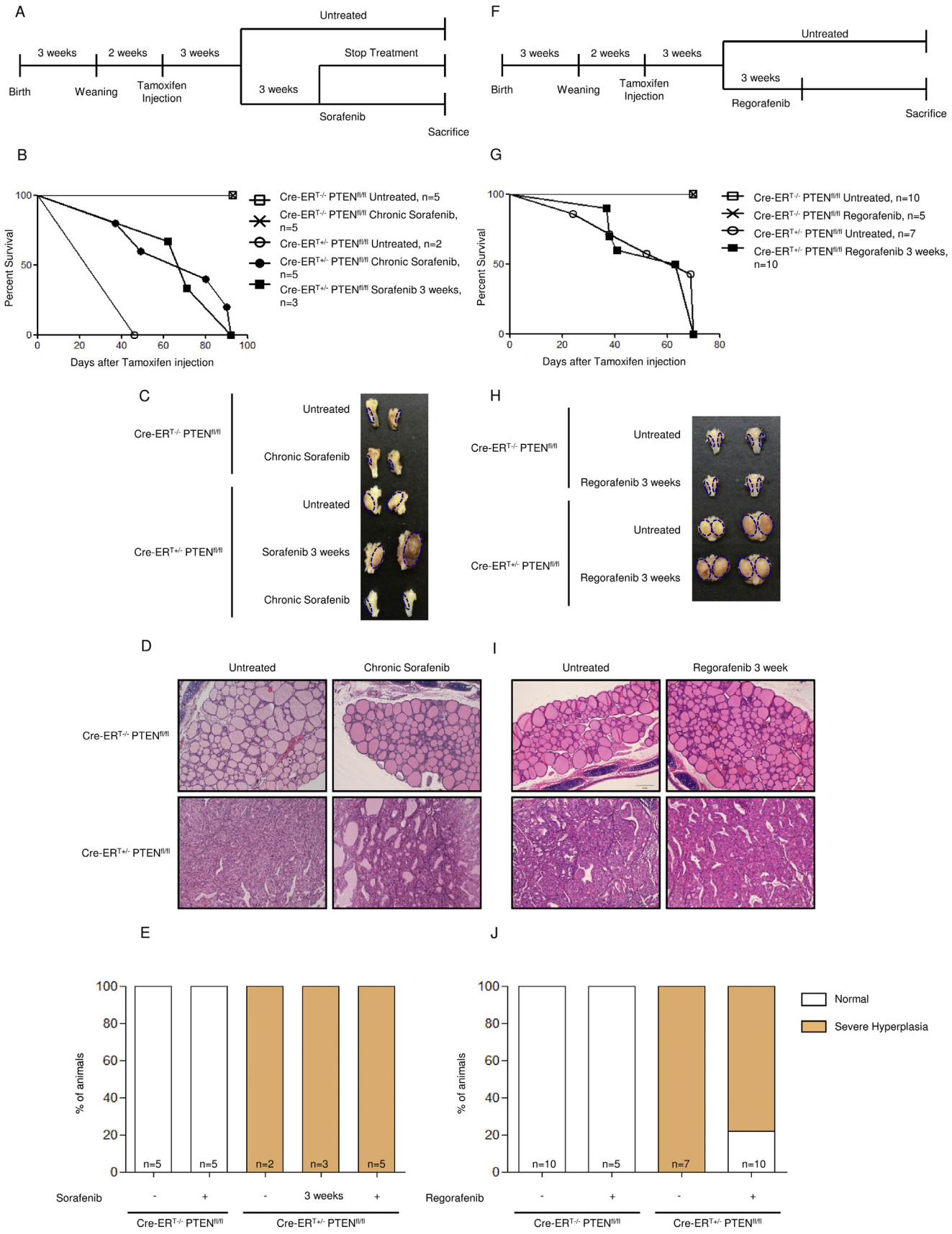


Fig. 6. Effects of chronic treatment with multikinase inhibitors in PTEN-deficient prostate neoplasias. (A) Macroscopic images of thyroids from Sorafenib-treated mice. (B and C) Representative images of HE staining (×10) and evaluation of lesions of prostates from Sorafenib-treated animals. Sorafenib induced a significant reduction of prostatic lesions. (D) Macroscopic images of thyroids from Regorafenib-treated mice. (E and F) Representative images of HE staining (×10) and evaluation of lesions of prostates from Regorafenib-treated animals. Regorafenib treatment had no effect on prostate neoplasias. Abbreviations: HE, haematoxylin and eosin; PTEN, phosphatase and tensin homolog deleted on chromosome 10.

their therapeutic potential depends on the tissue that needs to be treated.

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Conflict of interest statement

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejca.2016.04.019.

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