Introduction

Renal cell carcinoma (RCC), the 13th most common malignancy worldwide, kills about 116,000 people annually (1). While early-stage RCC is treatable by surgical and other interventions, the metastatic form of this malignancy is chemotherapy-resistant and lethal (2). RCC comprises several distinct histologic varieties, of which clear cell (cc)RCC represents the dominant subtype and accounts for up to 85% of all RCC cases (3). Over the past decade, small-molecule therapies that target growth factor-, angiogenesis-, and nutrient-sensing pathways (e.g., the tyrosine kinase inhibitors sunitinib and sorafenib) have become the frontline treatment options for advanced RCCs (2, 4). Although most patients will derive some benefit from these agents, virtually all will experience ultimately succumb to metastatic disease within 5 years (5, 6). Treatment of advanced RCCs is therefore still a significant therapeutic challenge.

Before the introduction of small-molecule targeted agents, cytokine-based immunotherapy with either IFN-α or high-dose interleukin (IL)-2 was the most common treatment option for metastatic RCCs (7). On the basis of its similarity to IFN-α, the cytokine IFN-γ was evaluated in several clinical trials for RCCs (8). In particular, 2 key phase I/II trials using low-dose IFN-γ produced overall response rates of 15% to 30% and complete response rates of up to 10% in metastatic RCCs, highlighting the ability of this cytokine to provide long-term remission even after termination of treatment (9, 10). On the basis of these encouraging findings, a large multicenter phase III trial using IFN-γ as a monotherapy for RCCs was conducted, but this trial found no significant difference between IFN-γ and placebo in overall response rates, time to disease progression, or median survival (11). As a result, IFN-γ was not pursued much further as a potential anti-RCC biotherapeutic.

It is noteworthy that the trials described above—including the disappointing phase III trial—were designed primarily to exploit the indirect immunomodulatory effects of IFN-γ (i.e., its capacity to stimulate the immune response to RCCs, without necessarily acting on the tumor itself). We suggest that a major advantage of IFN-γ over current small-molecule approaches is its pleiotropic nature: IFN-γ is not only a powerful activator of the antitumor immune response but is also anti-angiogenic and directly tumoricidal to susceptible cells. Emphasizing the immunomodulatory effects of IFN-γ at the expense of its other direct
antitumor properties (e.g., its anti-angiogenic and growth-suppressive effects) may have contributed to the failure of the phase III clinical trial. We are therefore focused on resurrecting IFN-γ as an anti-RCC therapeutic by exploiting its direct antineoplastic properties, and, specifically, its capacity to selectively kill tumor cells. To this end, we have recently shown that the transcription factor NF-xB activates a survival program that protects mammalian cells from IFN-γ (12). In the absence of this survival program, we found that IFN-γ activates a novel process of caspase-independent necrotic cell death [sometimes termed "necroptosis" (ref. 13)], mediated by the kinase RIP1 (12). As NF-xB drives a well-described survival program in many tumors—including RCCs (14–16) and as dividing cells were found to be especially susceptible to IFN-γ–induced necrosis (12), these discoveries readily lend themselves to exploitation for the treatment of RCCs.

One mechanism by which the small-molecule proteasome inhibitor bortezomib (PS-341, Velcade) functions as an antineoplastic agent is by inhibiting NF-xB (17), and studies have shown that blocking NF-xB with bortezomib in RCC cells (i) sensitizes them to the pro-apoptotic effects of TNF-α and TRAIL (18–20), (ii) synergistically potentiates the tumoricidal capacity of EGF receptor (EGFR) inhibitors (21), and (iii) increases susceptibility to oncolysis by encephalomyocarditis virus (22). In this study, we took advantage of the NF-xB inhibitory capacity of bortezomib to test whether blocking NF-xB signaling in RCCs rendered them susceptible to IFN-γ–induced necrosis.

Using a panel of patient-derived ccRCC cell lines, we report that inhibiting NF-xB by bortezomib renders RCC cells selectively susceptible to IFN-γ–induced necrosis. IFN-γ–triggered necrotic death was found to be independent of von Hippel-Lindau (VHL) gene or protein status and required the kinase RIP1. All RCC cells contained readily detectable basal NF-xB activity, comprising chiefly canonical RelA-P50 dimers, and bortezomib sensitized these cells to IFN-γ, in part, by inhibiting the prosurvival signaling activity of these NF-xB complexes. NF-xB signaling components and prosurvival target genes displayed elevated expression in samples from RCC tumors, compared with surrounding normal renal tissue, suggesting that RCC cells are more reliant on NF-xB survival signaling than their normal counterparts. In agreement, IFN-γ—in the setting of NF-xB inhibition by bortezomib—selectively induced necrosis in a panel of American Type Culture Collection (ATCC)-derived RCC cell lines at doses that were largely nontoxic to normal kidney epithelial cells. Collectively, these results suggest that the combination of IFN-γ and bortezomib will have therapeutically beneficial effect in RCCs.

Materials and Methods

Cell Lines

ccRCC cell lines HRC31, HRC45, and HRC63 were established at the Fox Chase Cancer Center (FCCC) as described previously (23). These cells were maintained in IIA medium [Dulbecco’s modified Eagle medium (DMEM):F12 supplemented with 1.2 g/mL NaHCO₃, 1.6 μmol/L FeSO₄, 50 mmol/L sodium selenite, 25 μg/mL insulin, 200 nmol/L hydrocortisone, 10 μg/mL transferrin, 1 mmol/L triiodothyronine, 10 μU/mL vasopressin, 10 nmol/L cholesterol, 10 ng/mL EGF, and 15% FBS]. ACHN, Caki-1, 786-O, and RenCa were obtained from the ATCC and cultured in DMEM/10% FBS (AHCN and Caki-1) or RPMI-1640/10% FBS (786-O and RenCa). Normal kidney epithelium–derived cell populations NKC1 and NKC2 were generated from minced renal tissue obtained from FCCC patients and cultured in ACL-4 medium [DMEM:F12 supplemented with 0.02 mg/mL insulin, 0.01 mg/mL transferrin, 25 nmol/L sodium selenite, 50 nmol/L hydrocortisone, 1 ng/mL EGF, 0.01 mmol/L ethanolamine, 0.01 mmol/L phosphorleythanolamine, 100 pmol/L triiodothyronine, 0.5% (w/v) bovine serum albumin, 0.5 mmol/L sodium pyruvate, 2 mmol/L L-glutamine, and 15% FBS]. ATCC cell lines were not authenticated in-house but were used within 6 months of resuscitation.

Reagents

Cytokines and chemicals were from the following sources: human IFN-γ (Pestka Biomedical Laboratories), Necrostatin-1 (Enzo Life Sciences), bortezomib (Millenium), IMD-0354 (EMD Biosciences). Oligonucleotides used to prepare radiolabeled wild-type and mutant NF-xB probes for electrophoretic mobility shift assay (EMSA) were purchased from Santa Cruz, whereas oligonucleotides used to make the gamma-activated sequence (GAS) probe were custom-synthesized at the FCCC. All other reagents were from Sigma-Aldrich, unless otherwise mentioned.

Antibodies

Primary antibodies for use in immunohistochemical and immunoblotting studies were purchased from Santa Cruz (pVHL, IRF-1, 1-xBt, IKKβ, P50, P52, RelA, c-Rel), Upstate (STAT1, pSTAT1 Y701, pSTAT1 S727), BD Biosciences (HIF-1α, RIP1), Ventana (cytokeratin, RCC marker), Dako (vimentin), Novocastra (CD10), and Novus (HIF-2α).

EMSA

EMSA reactions were conducted as described previously (24). Briefly, cells (2 × 10⁶/condition) were lysed hypotonically and nuclear extracts prepared by high-salt extraction. Ten micrograms of nuclear protein was incubated with a radiolabeled NF-xB probe derived from the HIV-1 long terminal repeat (5'-AGTTGAGGGACT-TTCCAGGC-3') (25), mutant NF-xB probe (5'-AGTTGAGGGACTTTCCAGGC-3'), GAS probe derived from the human IRF1 gene (5'-GATCGATTTCCGCCGAAAT-3'), and reactions resolved by 5% nondenaturing PAGE. Gels were then vacuum-dried and subjected to autoradiography. For antibody supershift experiments,
antibodies (1 μg) were added to nuclear extracts 15 minutes before incubation with radiolabeled oligonucleotide.

**RNA interference**

RCC cells (6 × 10^4/well) seeded into 6-well dishes were transfected with pools of 4 distinct proprietary siRNAs (SMARTpool, Dharmacon) to RIP1 at 20 nmol/L using Oligofectamine (Invitrogen) as a transfection reagent. As controls, nontargeting siRNA duplexes (Dharmacon) were used. Cells were used in experiments 48 to 72 hours posttransfection.

**Real-time quantitative PCR**

Cells (2 × 10^6/culture) were harvested in TRI Reagent (Applied Biosystems), and total RNA was extracted by phase separation in bromochloropropane (Molecular Research Center). RNA was reverse-transcribed into cDNA according to the manufacturer’s protocol (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems). Real-time quantitative PCR was carried out on an ABI7000 System using the Fast Start Universal Probe Master Mix (Roche), with probe and primer sets designed and supplied by the Roche Universal Probe Library System.

**Cell viability**

Cell viability was measured by Trypan Blue exclusion analysis. As necessary, necrosis was established by rescue of viability with Nec-1 (50 μmol/L) pretreatment.

**Statistical analysis**

The Student t test was used for comparison between 2 groups, and P < 0.05 was considered significant.

**Results**

**Characterization of three patient-derived ccRCC cell lines**

Three ccRCC cell lines, designated HRC31, HRC45, and HRC63, were established from tumor biopsies of patients undergoing surgery at the FCCC (23, 26). Each cell line stained positive in immunohistochemical studies with antibodies to cytokeratin, vimentin, CD10, and RCC marker, confirming ccRCC diagnosis (Fig. 1A).

The VHL gene, a key tumor suppressor in ccRCCs (27), was not mutated in any of the 3 cell lines (not shown), and its product—pVHL—was expressed in 2 of 3 cell lines (HRC31 and HRC45) at levels equivalent to those seen in 2 independent populations of cells cultured from normal renal epithelium (herein designated...
NC1 and NKC2 (Fig. 1B). Although VHL was unmutated in HRC63, these cells did not express detectable pVHL protein, suggestive of VHL inactivation in this cell line by epigenetic or posttranscriptional mechanisms (Fig. 1B). It is noteworthy that VHL mutations on average are found to only occur in approximately half of ccRCC samples (with a range of ~20% to ~70%, depending on the study), so unmutated VHL in all 3 RCC cell lines is not a statistical improbability.

Interestingly, although HRC31 and HRC45 each display normal pVHL levels (Fig. 1B) and 2 copies of chromosome 3p (where VHL resides), both cell lines showed LOH for all or part of chromosome 3p, based on allele profiling (Supplementary Fig. S1), indicative of uniparental isodisomy, a mechanism for copy-neutral LOH (32). The LOH included the region 3p21-p25, which, besides harboring several other tumor suppressor loci implicated in ccRCC tumorigenesis including PBRM1, SETD2, and BAP1 (31). Such LOH may unmask recessive mutations in the remaining allele of one or more of these tumor suppressor genes, providing a possible explanation for the normal VHL status of these 2 cell lines.

The VHL gene product (pVHL)—in its best-described role—functions by controlling the protein levels of hypoxia-inducible factor (HIF)-α, a master regulator of the cellular response to hypoxia (33). There are 3 HIF-α family members, HIF-1α, HIF-2α, and HIF-3α, of which HIF-1α and HIF-2α are particularly important in renal cell carcinogenesis (27). HIF-1α accumulated to robust levels in 2 of 3 cell lines (HRC45, HRC63), and HIF-2α in the third cell line (HRC31) even under normoxic conditions, in concordance with their roles as drivers of ccRCC tumorigenesis. Thus, all 3 RCC cell lines display elevated HIF-α expression despite unmutated VHL and (at least in the cases of HRC31 and HRC45) detectable pVHL protein.

As the exploitation of the direct tumoricidal properties of IFN-γ in RCCs is dependent on the capacity of RCC cells to support signaling downstream of the IFN-γ receptor, we next tested key aspects of classical IFN-γ signaling in the 3 RCC cell lines. The major IFN-γ-initiated signaling axis proceeds via Janus-activated kinase (Jak)1/2-mediated phosphorylation of the transcription factor STAT1, which then translocates to the nucleus and transactivates expression of IFN-stimulated genes (ISG) containing GAS elements in their promoters (34). In all 3 cell lines, IFN-γ induced robust phosphorylation of STAT1 on the key Y701 and S727 residues within 30 minutes of treatment (Fig. 1C), and a subsequent EMSA experiment revealed that nuclei from all 3 RCC cell lines contained IFN-γ-inducible activity capable of binding GAS elements, albeit with cell line–specific differences in kinetics and magnitude (Fig. 1D). Supershifting the IFN-γ-activated band with an anti-STAT1 antibody showed that the IFN-γ-inducible activity contains STAT1. In accordance with these observations, IFN-γ treatment upregulated protein levels of the prototypic ISG product IRF-1 in each RCC cell line (Fig. 1E). HRC45 displayed somewhat reduced GAS activity and IRF-1 induction, consistent with the modestly lower levels of total STAT1 protein seen in these cells. Together, these findings indicate that all 3 RCC cell lines are capable of mounting a STAT1-dependent transcriptional response following IFN-γ stimulation, a prerequisite for activation of necrosis by this cytokine.

Bortezomib and IKK inhibition sensitizes ccRCC cell lines to IFN-γ-induced cell death

We had previously reported that neutralizing NF-κB signaling sensitized dividing cells to IFN-γ-triggered necrotic death (12). As a start to exploiting this observation for the treatment of RCCs, we took advantage of the fact that a major mechanism by which the proteasome inhibitor bortezomib exerts its antitumor effects is via inhibiting NF-κB activation (17, 35). We first conducted dose-finding studies to identify doses of bortezomib that, on their own, were relatively nontoxic to each cell line (i.e., >85% cell viability in the continuous presence of that dose over 72 hours; Fig. 2B). We designated the highest such dose of bortezomib as the maximum tolerated dose (MTD, typically in the 2.5–5 nmol/L range) for the purposes of these studies and used that dose in subsequent experiments (Fig. 2A, arrows).

We next treated each RCC cell line with its MTD of bortezomib, following which we incubated them with increasing amounts of IFN-γ (up to 100 U/mL, well within the therapeutic range for this cytokine [ref. 36]). In control experiments, we treated parallel populations of RCC cells with the IKKβ inhibitor IMD-0354, before exposing them to these same doses of IFN-γ. IFN-γ alone was largely nontoxic to RCC cells, but induced progressive, dose-dependent cytotoxicity in the presence of either bortezomib or IKKβ inhibitor (Fig. 2B). In HRC31 and HRC63, the combination of IFN-γ and bortezomib was cytotoxic to more than 80% of cells within 72 hours, whereas HRC45 was somewhat more resistant (~60% loss of viability in the same time frame; Fig. 2B and C), perhaps as a result of its modestly poorer responsiveness to IFN-γ stimulation (see Fig. 1). Of note, about 100% of all 3 cell lines eventually succumbed to this combination 96 to 120 hours posttreatment, but the interpretation of these results are somewhat confounded by issues of toxicity arising from such prolonged exposure to bortezomib alone (data not shown). Collectively, these results show that bortezomib sensitizes RCC cells to IFN-γ-induced cell death at therapeutically achievable doses for both agents.

IFN-γ induces RIP1 kinase–dependent necrosis in ccRCC cells when NF-κB is inhibited

Necrosis in NF-κB–deficient MEFs exposed to IFN-γ resulted from accumulation of ROS in mitochondria and required the kinase RIP1 (12). Mechanistically, we have found that IFN-γ transcriptionally activated RIP1, which then impinged on mitochondria to hyperactivate ROS production and induce respiratory failure (12). To test whether a similar RIP1-dependent necrotic mechanism accounted for IFN-γ–triggered cytotoxicity in IKKβ inhibitor- or bortezomib-treated RCC cells, we first verified...
that each RCC cell line expressed RIP1 protein (Fig. 3A). We then incubated the RCC cell lines with the potent, specific RIP1 kinase inhibitor Necrostatin-1 (Nec-1; ref. 37), before exposing them to IFN-γ in the presence of either the IKKβ inhibitor (Fig. 3B) or bortezomib (Fig. 3C). Nec-1 afforded significant protection against IFN-γ–induced cell death in all 3 cell lines, rescuing each of them by at least 30% (Fig. 3B and C). Of note, the caspase inhibitor zVAD provided modest protection (up to 15%, depending on the cell line) against the combination of bortezomib and IFN-γ, suggesting that both necrotic and caspase-dependent apoptotic mechanisms contribute to IFN-γ–triggered cytotoxicity in these cells (data not shown).

To confirm the role of RIP1 in IFN-γ–driven cell death, we used RNA interference (RNAi) to silence RIP1 expression by more than 75% in each of the 3 RCC cell lines (Fig. 3D, inset), following which we treated these cells with IFN-γ in the presence or absence of bortezomib. In all 3 cell lines, RIP1 RNAi—like Nec-1 pretreatment—provided significant protection against the combination of IFN-γ and bortezomib (Fig. 3D). These data show that RIP1–dependent necrosis is a dominant mechanism of cell death induced by IFN-γ in the presence of bortezomib.

**Bortezomib inhibits NF-κB in ccRCC cells**

These results show that bortezomib and NF-κB inhibition can both sensitize RCC cells to necrosis induced by IFN-γ, suggesting that bortezomib—a known NF-κB inhibitor—functions, at least in part, by blocking constitutive NF-κB activity in ccRCC cells. To test whether bortezomib inhibited NF-κB in RCC cells, we first
determined by EMSA that all 3 RCC cell lines displayed detectable basal NF-κB activity (Fig. 4A). Supershift studies using antibodies to NF-κB subunits revealed that the primary NF-κB complexes in all 3 cell lines comprised chiefly canonical RelA:P50 heterodimers, although dimeric combinations of other subunits (e.g., P52- and c-Rel–containing complexes in HRC45) may also exist in lesser amounts (Fig. 4A).

Next, we incubated each RCC cell line with bortezomib for 12 hours, following which we subjected nuclear extracts from these cells to NF-κB EMSA. As shown in Fig. 4B, bortezomib treatment significantly reduced basal NF-κB activity in all 3 RCC cell lines. By densitometry, we determined that bortezomib treatment reduced NF-κB DNA–binding activity by ~50% (HRC31), ~90% (HRC45), and ~80% (HRC63) in the 3 RCC cell lines (Fig. 4C). DNA binding to a control Oct-1 probe (not shown) or RelA/P50 protein levels (not shown) were not significantly affected by bortezomib treatment showing that bortezomib specifically inhibits NF-κB activity in RCC cells. In agreement with these findings, bortezomib efficiently prevented degradation of IκBα following acute exposure of HRC63 cells to the well-established NF-κB stimulator TNF-α (Supplementary Fig. S2). As a consequence, bortezomib was as effective as an IKKβ inhibitor in sensitizing HRC63 cells to TNF-α–induced cell death (Supplementary Fig. S2).

We note, however, that while bortezomib can inhibit constitutive NF-κB signaling in RCC cells, the degree to which it does so (particularly in HRC31) does not fully correlate with its ability to sensitize cells to IFN-γ. Thus, bortezomib likely engages multiple proteasome-sensitive mechanisms to sensitize RCC cells to IFN-γ.

NF-κB prosurvival target gene expression is inhibited by bortezomib and elevated in ccRCC

We have previously reported that the NF-κB target SOD2 (encoding the antioxidant enzyme MnSOD) is necessary for protection against IFN-γ–induced necrosis (12). To test whether bortezomib inhibited MnSOD expression in the 3 RCC cell lines, we treated these cells with IFN-γ and examined MnSOD levels in lysates from these cells.
As shown in Fig. 5A, we found that bortezomib inhibited both basal and IFN-γ-induced expression of MnSOD in all 3 RCC cell lines.

To extend these observations, we analyzed the mRNA expression profiles of SOD2, as well as of 2 additional mitochondrial prosurvival targets of NF-κB—the Bcl-2 family member Bfl-1 (encoded by BCL2A1) and the antioxidant enzyme Ferritin Heavy Chain (FHC, encoded by FTH1)—following treatment of RCC cell lines with bortezomib. Like MnSOD, both Bfl-1 and FHC have been reported to protect cells from cytokine-driven mitochondrial damage and cell death (38, 39). We found that bortezomib significantly downregulated the expression of each of these mRNAs in all 3 cell lines (Fig. 5B).

While these results support the idea that bortezomib will sensitize RCC cells to IFN-γ-induced necrotic death by blocking NF-κB survival signaling (e.g., by downregulating MnSOD levels) in vivo, a precondition for the specific killing of RCC—but not normal—cells by the combination of IFN-γ and bortezomib (or other NF-κB blockers) is that ccRCC cells, compared with normal kidney tissues, must selectively display an increased reliance on NF-κB–dependent survival mechanisms. Data suggest that established ccRCC cell lines do indeed display constitutively elevated NF-κB activity (16, 19, 21, 40, 41) and that increased NF-κB activity correlates with progression of disease in patients (42).

Elevated NF-κB activity, however, is not necessarily indicative of increased prosurvival gene induction, and whether dedicated NF-κB prosurvival targets are upregulated in ccRCCs has not been determined. We therefore examined NF-κB prosurvival gene expression in publicly available datasets where ccRCCs and normal renal tissues were assessed for transcriptomic changes. In particular, we focused on raw expression data generated by Copland and colleagues (ref. 43; made available through GEO), as these data paired ccRCCs and normal renal cortical tissue obtained from the same patients. Analysis by Linear Models for Microarray Data (LIMMA; ref. 44) of the expression levels of the primary NF-κB signaling components (genes encoding IKKγ, P50, and RelA) and key downstream survival targets (genes encoding MnSOD, Bfl-1, and FHC) in this data set revealed that all these genes were significantly overexpressed in ccRCC samples, compared with their paired normal controls, strongly suggesting that elevated NF-κB survival signaling is a common feature of ccRCCs (Fig. 5C). Notably, very similar results were obtained when the larger (albeit less robustly controlled) dataset generated by Libermann and colleagues (45) was analyzed (Supplementary Fig. S3).

**Bortezomib sensitzes established RCC cell lines to IFN-γ–induced necrosis**

The elevated expression of NF-κB prosurvival targets—and, in particular, antioxidant-encoding genes like SOD2 and FTH1—in RCC samples suggests that RCC cells possess mitochondria that are metabolically more active than those found in surrounding normal tissue and are therefore more reliant on ROS-quenching antioxidant enzymes such as MnSOD and FHC for their survival. As IFN-γ–driven necrosis is a ROS-driven process, RCC cells, compared with normal kidney epithelial cells, will be expected to display selective susceptibility to IFN-γ when NF-κB is disabled (i.e., RCC cells are “addicted” to elevated NF-κB signaling). To test this prediction, we treated a panel of 4 ATCC-derived human (ACHN, Caki-I, and...
and murine (RenCa) RCC cell lines with the combination of IFN-γ and bortezomib. In parallel, we also treated 2 independent populations of cells from normal renal epithelium (NKC1 and NKC2) with the same combination. In line with the expectation that RCC cells are selectively addicted to elevated NF-κB activity for their survival, both NKC populations displayed undetectable basal NF-κB activity (not shown) and remained resistant to the combination of IFN-γ and bortezomib at concentrations of each agent (50–100 U/mL IFN-γ and 5 nmol/L bortezomib) that induced potent necrosis in all RCC cell lines tested (Fig. 6A). In fact, both NKC1 and NKC2 remained mostly viable (<10% cell death) for at least 72 hours in the continued presence of up to 10-fold higher concentrations of IFN-γ (i.e., 1,000 U/mL) or 2.5-fold higher concentrations of bortezomib (12.5 nmol/L) in subsequent dose-escalation experiments, when the concentration of one agent was held constant whereas that of the other was increased (not shown).

Collectively, these data show that elevated NF-κB activity can be exploited to selectively sensitize RCC cells to the pronecrotic effects of IFN-γ and provide strong rationale for the combined use of bortezomib (or other NF-κB inhibitors) with IFN-γ for the treatment of this malignancy.

Discussion

The ideal IFN-γ–based anti-RCC therapy will not only take advantage of the immunomodulatory activity of IFN-γ but also exploit this cytokine’s other antineoplastic properties—including its anti-angiogenic and direct tumoricidal capacity—and do so with minimal toxic side effects. With this goal in mind, we have identified NF-κB as one druggable mechanism by which RCC cells are
resistant to the direct tumoricidal properties of IFN-γ. We had previously shown that when NF-xB is disabled, IFN-γ transcriptionally activates a novel form of necrotic cell death (sometimes termed necroptosis) dependent on the kinase RIP1 (12). Here, we exploited these findings to show that the small-molecule proteasome inhibitor bortezomib sensitizes RCC cells to IFN-γ–induced necrotic death, at least in part, by blocking NF-xB.

How does NF-xB protect RCC cells from IFN-γ? Results from our previous work suggest that IFN-γ uses RIP1 to boost mitochondrial activity and ATP biogenesis—for example, to fuel ATP-dependent anti-microbial enzymes—during physiologic immune responses (12). An unavoidable consequence of increased mitochondrial activity is the generation of toxic reactive oxygen species (ROS), typically as a byproduct of leaky electron transport during the process of oxidative phosphorylation (46). Scavenging of ROS and other free radicals is a primary task of the NF-xB prosurvival program, as evidenced by the facts that (i) ROS accumulates to toxic levels following cytokine stimulation of NF-xB–deficient cells and (ii) overexpression of the NF-xB targets MnSOD and FHC quenches ROS after cytokine exposure without need for NF-xB activity (12, 39, 47). When ROS is allowed to accrue in mitochondria (e.g., following exposure to IFN-γ in settings of NF-xB inhibition), mitochondrial membrane potential is lost, respiratory failure occurs, and the cell dies by caspase-independent necrosis (12).

Although the process of tumorigenesis often results in a shift in ATP production from oxidative phosphorylation to aerobic glycolysis, oxidative phosphorylation is still thought to generate a significant fraction (from less than half to more than 90%, depending on the malignancy) of the tumor cell’s ATP needs (48, 49). We propose that the increased demand for ATP during tumorigenesis makes tumor cells more reliant on NF-xB–activated free radical scavenging mechanisms—both constitutively, and in response to acute stimuli such as IFN-γ—to ensure mitochondrial integrity and cell survival. Indeed, several observations lend support to the idea that tumor cells (including RCCs) have co-opted NF-xB prosurvival signaling to sustain their own viability. These include the findings that (i) high constitutive NF-xB activity in a frequent occurrence in diverse cancers, (ii) activating mutations in genes encoding NF-xB subunits have been reported in several tumor types, and (iii) tumor cells are often “addicted” to NF-xB survival signaling and succumb when NF-xB signaling is inhibited or ablated (14, 50).

Established RCC cell lines and patient tumor samples—as a result of CARD9 activation and/or chronic EGF receptor signaling—also display constitutively elevated NF-xB prosurvival activity (22, 26, 28). In agreement, our *in silico* analyses of published patient data reveal that NF-xB signaling components and downstream prosurvival target genes are chronically upregulated in RCC tumors (Fig. 5). It is particularly noteworthy that the NF-xB targets SOD2 (encoding manganese superoxide dismutase, or MnSOD) and *FTH1* (encoding ferritin heavy chain, FHC) were elevated in cRCCs, as both these gene products are antioxidant enzymes critical for NF-xB–dependent buffering of mitochondria against cytokines such as IFN-γ and TNF-α (12, 39, 47). Thus, when NF-xB is blocked in RCC (e.g., by bortezomib), antioxidant enzymes such as MnSOD and FHC are not induced, and consequent accrual of ROS and other free radicals following IFN-γ stimulation likely results in mitochondrial toxicity, respiratory failure, and eventual necrotic death (schematically depicted in Fig. 6B).

Collectively, these observations suggest that the combination of IFN-γ and NF-xB inhibition will have a...
therapeutic benefit in RCCs. NF-κB, however, is a master transcription factor with multiple functions, and agents that inhibit NF-κB therefore have numerous toxic side effects (50). The pleiotropic nature of the NF-κB transcriptional response is a major limitation to the development of new NF-κB antagonists, and—to the best of our knowledge—no dedicated NF-κB inhibitor has yet been approved for antitumor therapy (50). Although bortezomib mediates its antitumor effects, at least in part, by NF-κB blockade, it almost certainly sensitizes RCC cells to IFN-γ by mechanisms additional to simple inhibition of NF-κB prosurvival signaling, such as, for example, stabilization of pro-apoptotic proteins or induction of endoplasmic reticulum (ER) stress (17). We therefore suggest that determining how NF-κB specifically mediates cell survival (i.e., identifying druggable prosurvival targets of NF-κB) is essential to the development of next-generation NF-κB–targeted antitumor agents. Such agents, by selectively blocking NF-κB prosurvival signaling without affecting other aspects of NF-κB signaling, will be expected to minimize toxicity issues impacting current NF-κB inhibitors.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References


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