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Discovery of a Selective Irreversible BMX Inhibitor for Prostate Cancer

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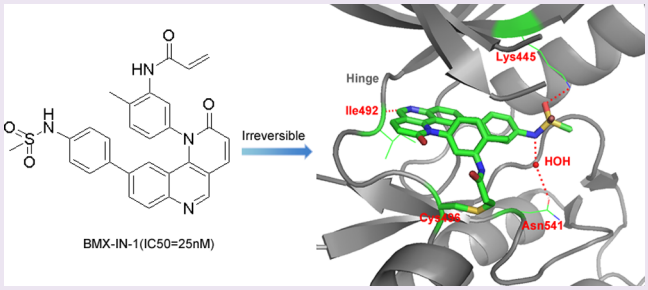
S *Supporting Information*

ABSTRACT: BMX is a member of the TEC family of nonreceptor tyrosine kinases. We have used structure-based drug design in conjunction with kinome profiling to develop a potent, selective, and irreversible BMX kinase inhibitor, BMX-IN-1, which covalently modifies Cys496. BMX-IN-1 inhibits the proliferation of Tel-BMX-transformed Ba/F3 cells at two digit nanomolar concentrations but requires single digit micromolar concentrations to inhibit the proliferation of prostate cancer cell lines. Using a combinatorial kinase inhibitor screening strategy, we discovered that the allosteric Akt inhibitor, MK2206, is able to potentiate BMX inhibitor's antiproliferation efficacy against prostate cancer cells.



BMX (also termed ETK) is a member of the TEC family of nonreceptor tyrosine kinases (which also includes ITK, TEC, BTK, and TXK) and is the major member of this family expressed in epithelial cells, including prostate epithelium. Similar to the SRC family of kinases, the TEC kinases contain core SH3, SH2, and kinase domains, but they are unique in having an N-terminal pleckstrin homology domain that mediates membrane recruitment through binding to phosphatidylinositol 3,4,5-triphosphate generated by phosphatidylinositol-3 kinase (PI-3K).[1−3] BMX is further activated by the subsequent phosphorylation of a tyrosine in its kinase domain by membrane-associated SRC. The activation of BMX in response to PI-3K signaling, which is increased in prostate cancer (PCa) due to PTEN loss, suggests a potential role for BMX in PCa. Indeed, BMX expression is increased in PCa, and transgenic overexpression of BMX in mouse prostate epithelium causes hyperplasia and contributes to development of dysplastic lesions resembling human prostate intraepithelial neoplasia (PIN).[4] BMX is also increased in castration-resistant prostate cancer (CRPC) and can enhance androgen receptor (AR) responses to low androgen levels.[5] Conversely, BMX down-regulation *in vitro* suppresses the growth of PCa cells. In addition, BMX expression is directly negatively regulated by AR, suggesting a role in resistance to androgen deprivation therapy.[5] In order to determine the pharmacological consequences of acute inhibition of BMX tyrosine kinase activity in PCa, we sought to develop potent and selective covalent inhibitors directed to the ATP-binding site.

In order to develop irreversible BMX inhibitors, we designed compounds capable of targeting cysteine 496, which is located at the lip of the ATP-binding site at a position equivalent to cysteine 797 of EGFR, which has been successfully targeted by numerous covalent EGFR inhibitors.[6] Kinome-wide sequence alignment reveals that there are ten kinases that have an equivalently positioned cysteine including all five TEC-family kinases, the catalytically active EGFR-family kinases (EGFR, Her2, and Her4), JAK3, and the Src-family kinase BLK.[7] There

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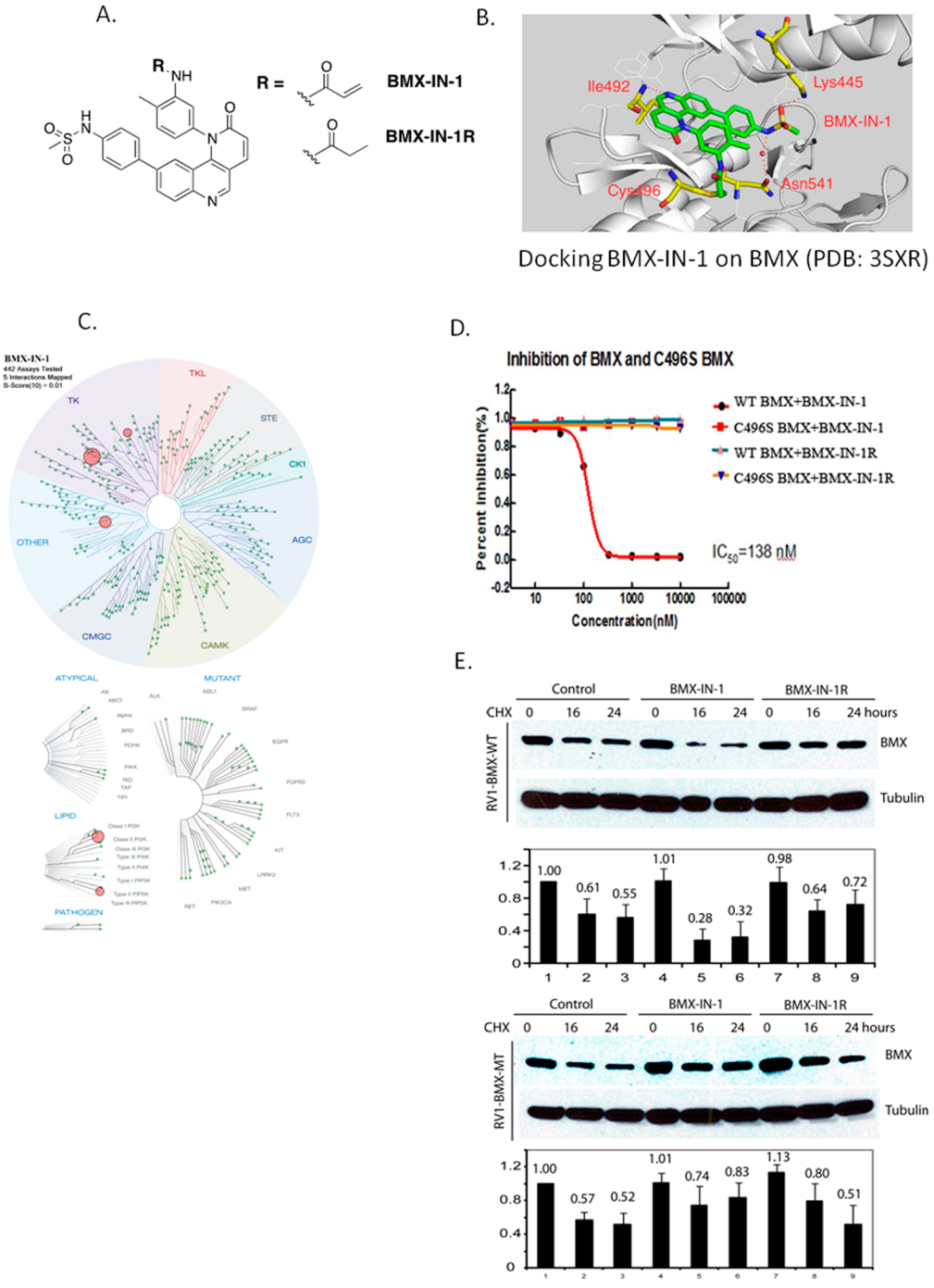

Figure 1. Characterization of BMX-IN-1 as an irreversible BMX inhibitor: A. chemical structure of BMX-IN-1 and BMX-IN-1R; B. predicted mode of binding of BMX-IN-1 to BMX based upon molecular modeling (PDB: 3SXR); C. TreeSpot view of the kinase selectivity profile of BMX-IN-1 using data generated from the KinomeScan approach; D. *in vitro* kinase assay using Flag-tagged BMX of either wild-type or C496S immunopurified from HEK293 cells showed that BMX-IN-1 inhibits only wild-type BMX with an IC_{50} of 138 nM, whereas BMX-IN-1R fails to inhibit both wild-type and C496S BMX; E. BMX-IN-1 (2.5 μM) induces degradation of wild-type but not C496S BMX in stably transfected RV-1 cells.

are a number of advantages to develop covalent inhibitors when seeking first-in-class inhibitors that are to be used as pharmacological tools to interrogate the functional consequences of inhibiting a particular kinase. First, suitably designed covalent inhibitors can be exceptionally potent and can often result in complete target inhibition in cell culture or in animal models with only transient drug exposure, which greatly reduces the need to extensively optimize pharmacokinetic properties. Second, covalent targeting of a particular cysteine residue provides another handle to achieve a high degree of kinase selectivity: noncovalent recognition only needs to be able to discriminate between the set of kinases that possess an equivalently positioned cysteine, while covalent bond formation can drive selectivity relative to all other kinases that do not possess an equivalently placed cysteine. Third, for irreversible inhibitors that require covalent bond formation to drive potency, a mutant form of the kinase can be engineered where the reactive cysteine is mutated to a nonreactive serine to

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provide a means to perform rescue experiments to validate that the observed pharmacological activity is indeed target-dependent.

In order to develop a covalent BMX inhibitor, we queried our database of kinase inhibitor selectivity profiles generated primarily using the KinomeScan approach for ATP-site-directed pharmacophores and searched for inhibitors that exhibited modest binding affinity and selectivity for BMX.[8] The search revealed that a subset of our tricyclic quinoline compounds, which we had been previously elaborated into highly potent inhibitors of mTOR, such as Torin1, possessed modest affinity (KinomeScan score of 59) for BMX.[8−11] We then used molecular modeling employing the published BMX crystal structure (PDB ID: 3SXR) to create a likely ATP-binding site pose for the inhibitor (Supplementary Figure 1). By comparing this model with our previous efforts to target an identically placed cysteine residue in the T790M mutant of EGFR with a pyrimidine-derived inhibitor (PDB ID: 3IKA), we were able to deduce a probable position to introduce an electrophilic acrylamide moiety targeting Cys496.[12] These efforts resulted in the design of BMX-IN-1, which was synthesized in seven steps as detailed in the Supporting Information (Figure 1A,B). BMX-IN-1 inhibited recombinant BMX kinase activity using the Z'lyte methodology with an IC_{50} of 8.0 nM. Selectivity profiling against a panel of 442 kinases using the KinomeScan approach at a concentration of 1 μM revealed that BMX-IN-1 exhibited remarkable selectivity with an S(10) score of 0.01[8] (Figure 1C and Supplementary Table 1). Enzymatic assays using SelectScreen on the kinases that possess an equivalently placed cysteine as BMX Cys496 revealed that BMX-IN-1 also potently inhibited BTK with an IC_{50} of 10.4 nM (Table 1). To

Table 1. Biochemical IC_{50}s of BMX-IN-1 Measured with Invitrogen SelectScreen Technology

kinase	BLK	BMX	BTK	JAK3	EGFR (T790M)	ITK	TEC
IC_{50} (nM)	377	8.0	10.4	175	4280	5250	653

determine whether BMX-IN-1 can selectively inhibit BMX kinase activity in a cellular context, we tested its ability to inhibit the proliferation of a panel of murine Ba/F3 cells that were transformed with TEL fusions of BMX, JAK1, JAK2, JAK3, TYK2, and BLK. The ability of inhibitors to block proliferation of oncogenic kinase-transformed Ba/F3 cells provides a commonly used means to establish the cellular activity and selectivity of kinase inhibitors.[13,14] Only the proliferation of Tel-BMX-transformed Ba/F3 cells was potently inhibited by BMX-IN-1 with an IC_{50} of 25 nM, demonstrating the ability of the drug to inhibit BMX in cells and to discriminate among kinases such as JAK3 and BLK, which possess an identically positioned reactive cysteine (Table 2).

We next sought to establish whether covalent bond formation to Cys496 was required for BMX-IN-1 to function as a potent cellular inhibitor of BMX. First, we synthesized an approximately isosteric analogue in which the electrophilic acrylamide is replaced with a nonreactive propyl amide to generate BMX-IN-1R (Figure 1A). BMX-IN-1R was over 400-fold less potent (IC_{50} > 10 μM) at inhibiting the proliferation of TEL-BMX-transformed Ba/F3 cells relative to BMX-IN-1 and also does not inhibit the biochemical kinase activity of BMX or BTK at concentrations below 10 μM. Second, we evaluated the ability of BMX-IN-1 to inhibit the activity of a mutant (Cys496Ser) BMX in which the reactive cysteine was mutated to a less reactive serine. We transiently transfected HEK293 cells with an expression vector of either wild-type or mutant Flag-tagged BMX and immunopurified the BMX kinases using anti-Flag antibody. The subsequent *in vitro* kinase assay revealed that BMX-IN-1 could potently inhibit wild-type BMX with an IC_{50} of 138 nM, while being incapable of inhibiting Cys496Ser BMX at concentrations below 10 μM (Figure 1D and Supplementary Figure 2). We also generated stably transformed Cys496Ser TEL-BMX Ba/F3 cells and confirmed that they were resistant to BMX-IN-1 (IC_{50} > 10 μM) (Supplementary Figure 3). Taken together, these results suggest that BMX-IN-1 requires covalent modification of Cys496 of BMX to achieve potent inhibition.

We next sought to probe the ability of BMX-IN-1 to inhibit the proliferation of a small panel of well-characterized prostate cancer cell lines, including RV-1, DU-145, PC-3, VCAP, and C4-2. In contrast to the low nanomolar concentrations of BMX-IN-1 required to inhibit the proliferation of the Ba/F3 cells that were engineered to be addicted to TEL-BMX kinase activity, the proliferation of prostate cancer cell lines was only inhibited by BMX-IN-1 in the single-digit micromolar range (Table 3). We chose to study in detail the effects of BMX-IN-1

Table 3. Antiproliferative Activity of BMX-IN-1 against a Panel of Prostate Cancer Cell Lines

cell line	RV-1	DU-145	PC-3	VCAP	C4-2
GI_{50} (μM)	2.54	4.38	5.37	2.46	>10

on RV-1 cells, as this is a commonly used prostate cancer cell line. The proliferation of RV-1 cells following a 5 days incubation with BMX-IN-1 was inhibited with an IC_{50} of 2.53 μM (Supplementary Figure 4). Consistent with proliferation being mediated by direct inhibition of BMX kinase activity, treatment of RV-1 cells with a 1 μM concentration of BMX-IN-1 was sufficient to inhibit BMX autophosphorylation (Supplementary Figure 5). Treatment of RV-1 cells with BMX-IN-1 at a concentration of 5 μM results in reduced cell numbers and induces apoptosis as assessed by Caspase 3 staining (Figure 2A). In contrast, the noncovalent analogue, BMX-IN-1R, did not possess antiproliferative effects against RV-1 cells at concentrations below 10 μM (Supplementary Figure 4). To establish whether these antiproliferative effects were dependent on inhibition of BMX, we attempted to rescue the drug effect by transfection of RV-1 cells with the inhibitor-resistant Cys496Ser mutant of BMX. Unfortunately, transfection of Cys469Ser BMX into RV-1 cells resulted in cells that did not proliferate well and that were therefore not suitable for 5 days drug treatment studies. These results suggest that BMX-

Table 2. Antiproliferative Activity of BMX-IN-1 against TEL-Kinase Transformed Ba/F3 Cell Lines

drug	Wt-Ba/F3	TEL-BMX	TEL-JAK1	TEL-JAK2	TEL-JAK3	TEL-TYK2E957D	TEL-BLK
BMX-IN-1 (GI_{50}: μM)	>10	0.025	4.92	5.83	7.98	6.09	3.64
BMX-IN-1R (GI_{50}: μM)	>10	>10	>10	>10	>10	>10	>10

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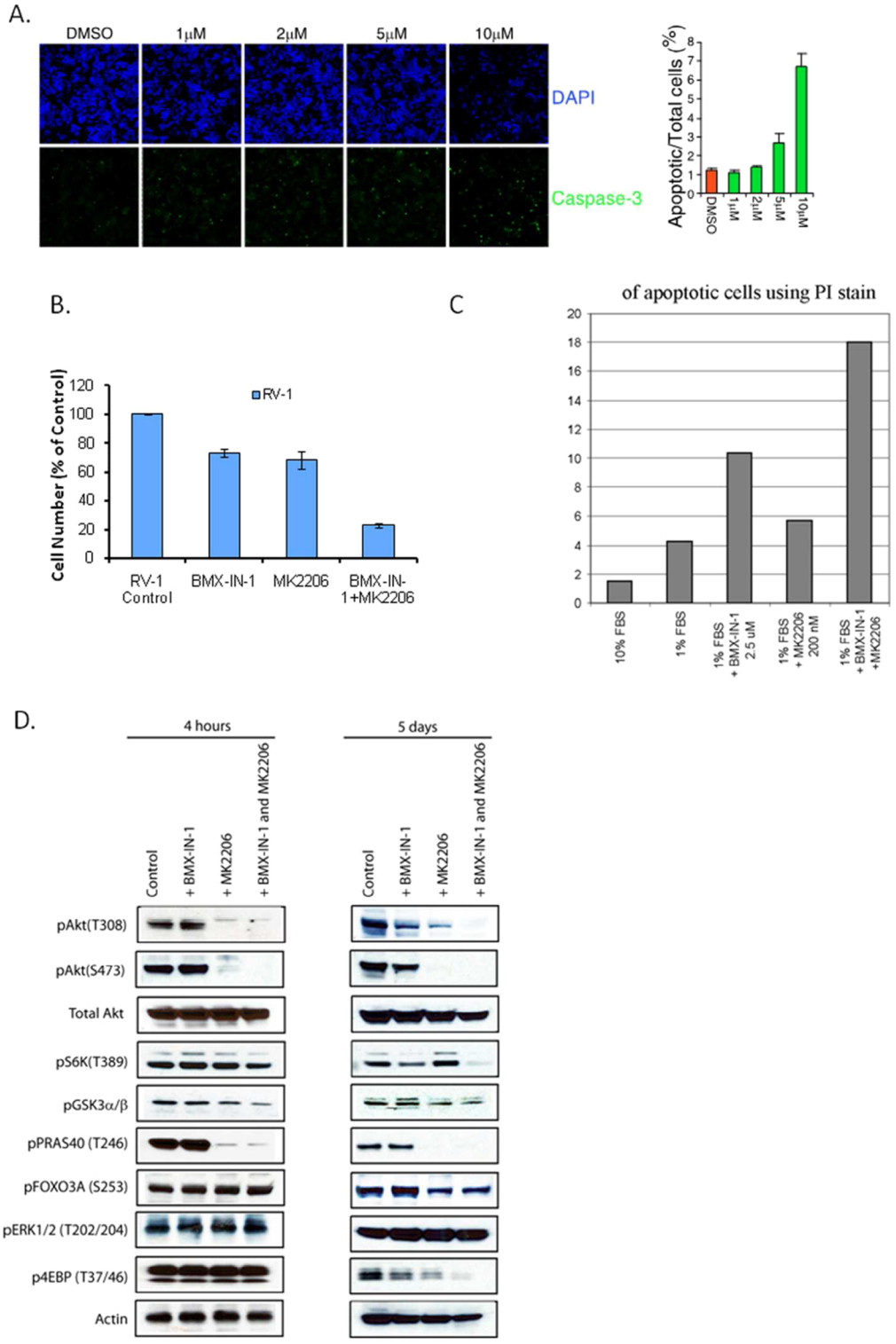

Figure 2. Effect of BMX-IN-1 on prostate cancer cells: A. BMX-IN-1 induces RV-1 cell apoptosis; B. combination study, BMX-IN-1 (2.5 μM) synergizes with the Akt inhibitor MK2206 (200 nM); C. flow cytometry analysis of the drug combination effect on apoptosis; D. effect of short and long-term combinatorial drug treatment on RV-1 cells: BMX-IN-1 (2.5 μM) and MK2206 (200 nM).

IN-1 exhibits antiproliferative effects that are dependent upon the acrylamide functional group but are inconclusive in regards to whether this effect is derived from inhibition of BMX.

We next investigated whether treatment of RV-1 cells with BMX-IN-1 affected BMX protein levels using RV-1 cells stably transfected with both wild-type and C496S mutant BMX. BMX protein levels in RV-1 cells were significantly reduced by a 72 h treatment of BMX-IN-1 (5 μM) but not BMX-IN-1R (Supplementary Figure 6). With blockage of nascent protein synthesis using cyclohexamide, the level of ectopically expressed BMX protein in RV-1 cells was observed to decrease upon treatment with BMX-IN-1 as compared to the control and treatment with BMX-IN-1R (Figure 1E). Moreover, the depletion could be rescued upon stable overexpression of the

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C496S mutant BMX. These results indicate that, in addition to inhibiting BMX catalytic activity, BMX-IN-1 can also decrease BMX protein levels.

Considering the relatively modest antiproliferative effects of BMX-IN-1 against RV-1 and other prostate cancer cell lines, we hypothesized that selective inhibition of BMX may not be sufficient to inhibit proliferation of prostate cancer cells. Consequently, we initiated a combinatorial screening effort to identify other kinases whose inhibition might potentiate the antiproliferative activity of BMX-IN-1. We assembled a 200-member kinase inhibitor library containing all approved and many clinical stage inhibitors of a wide range of protein kinases (https://lincs.hms.harvard.edu). We first screened the library to identify which compounds could inhibit the proliferation of RV-1 cells as single agents at a concentration of 200 nM. We then performed a screen with BMX-IN-1 fixed at concentrations of 500, 1000, and 2500 nM in combination with each in the 200-membered kinase inhibitor library. Several kinase inhibitors from the library appeared to potentiate the antiproliferative activity of BMX-IN-1, including inhibitors of mTOR (AZD8055, Torin1, Torin2, and WYE125132), PI3K (GDC0941), EGFR and Her2 (erlotinib, gefitinib, and lapatinib), and the allosteric Akt inhibitor (MK2206) (Figure 2B and Supplementary Figure 7). As the PI3K/AKT signaling pathway is activated in prostate cancer cells, coupled with the fact that BMX is activated in response to PI-3K signaling, we decided to investigate the combination of BMX-IN-1 with the allosteric Akt inhibitor, MK2206, in more detail. MK2206 is a very selective Akt inhibitor that does not inhibit BMX kinase activity at concentrations below 10 μM. Dose–response experiments demonstrated that concentrations as low as 25 nM of MK2206 could potentiate the antiproliferative activity of BMX-IN-1 against RV-1 cells (Supplementary Figure 8). Fluorescence activated cell sorting (FACS) using propidium iodide (PI) staining demonstrated that the inhibitor combination increased apoptosis as assessed by the percentage of sub-G1 cells without exerting major effects on the cell cycle distribution (Figure 2C and Supplementary Figure 9). We next examined the effects of single agent and combinatorial treatment on signaling by examining the phosphorylation status of known effectors of the PI3K-Akt-mTOR (Akt, S6K, Gsk3β, PRAS40, 4EBP, and FOXO3A) and MAPK (Erk1/2) pathways following acute (4 h) and long-term (5 day) treatments (Figure 2D). Acute treatment of RV-1 with BMX-IN-1 at 200 nM did not decrease the phosphorylation of any effectors, while long-term treatment resulted in substantial inhibition of phosphorylation of PI3K-pathway effectors, including Akt (T308), S6K, and 4EBP. In contrast, acute treatment of RV-1 with MK2206 resulted in substantial inhibition of phosphorylation of Akt and PRAS40, while long-term treatment maintained this inhibition and also resulted in dephosphorylation of 4EBP and S6K. Acute combination treatment resulted in a dephosphorylation profile mimicking single agent MK2206 treatment; however, long-term treatment resulted in more profound inhibition of most effectors. Especially notable were effects on S6K and 4EBP, reminiscent of what was observed following inhibition of mTOR with ATP-competitive mTOR inhibitors.

An accumulating body of literature suggests that BMX could be a potential therapeutic target in prostate and other cancers.[2,5] For example, RNAi knockdown of BMX causes an antiproliferative effect on prostate cancer cell lines and in animal models.[4] However, to date, there have been no selective BMX inhibitors developed, and there is a limited understanding of physiological or pathological functions of BMX. Here, we used kinome-wide screening and structure-based design to prepare BMX-IN-1, a potent and selective covalent inhibitor of BMX and BTK. A comprehensive kinase selectivity profiling suggests that the compound is quite selective although additional targets, including nonkinases, may be revealed through additional experiments. BMX-IN-1 covalently targets cysteine 496 using its acrylamide moiety, and this modification is required to achieve a potent inhibition of BMX-dependent cellular inhibition. BMX-IN-1 also potently inhibits BTK, which is primarily expressed in the B-cell, and would not be expected to confound using BMX-IN-1 as a pharmacological probe of BMX function in endothelial and epithelial cells.[1] Interestingly, we also discovered that BMX-IN-1 not only inhibits BMX kinase activity but also induces BMX degradation, providing a potential mechanism for antagonizing nonkinase dependent BMX functions.[3] The mechanistic basis for this inhibitor-induced degradation and its biological implications are currently being investigated.

In contrast to the highly potent inhibition of proliferation of cells engineered to be addicted to BMX activity, the proliferation of prostate cancer cell lines is only blocked at single digit micromolar concentrations of BMX-IN-1. This suggests that the degree of dependency on BMX kinase activity of prostate cells grown in cell culture is substantially less than Tel-BMX transformed Ba/F3 cells. Although the mechanistic basis for this is unclear, it is plausible that there may be dynamic signaling compensation following BMX inhibition. Of relevance, we demonstrated that the antiproliferative potential of BMX-IN-1 against RV-1 cells could be potentiated with other targeted kinase inhibitors, such as the allosteric Akt inhibitor, MK2206.

Ibrutinib (PCI-32765), a reported irreversible inhibitor of BTK, is also a potent inhibitor of BMX with a reported biochemical IC_{50} of 800 pM but also potently inhibits most TEC-family kinases.[15] Ibrutanib is currently being developed for B cell malignancies and has shown promise in early clinical trials.[16] A phase III study of ibrutinib versus ofatumumab in patients with relapsed or refractory chronic lymphocytic leukemia (CLL) is ongoing. However, despite its broadened kinase selectivity profile relative to BMX-IN-1, both compounds exhibit similar potency for inhibiting RV-1 cell proliferation (Supplementary Figure 10).

As prostate cancer is poorly modeled by prostate cancer cell lines grown in culture, additional experiments using stromal supported culture, murine tumor models, and ultimately clinical investigation will be required to address whether BMX is a therapeutically useful target. We anticipate BMX-IN-1 will be a useful addition to the growing arsenal of selective kinase inhibitors that can be used to delineate the role of BMX in health and disease.

METHODS

Chemical synthesis, antibodies, mutagenesis, IP kinase assays, proliferation assays, immunoblotting, and molecular modeling are described in detail in the Supporting Information section. It should be noted that all of the cell proliferation studies with BMX-IN-1 and the AKT inhibitor were done using low serum conditions (1% FBS).

Protein Degradation Assay. RV1-BMX-WT (wild-type) and RV1-BMX-MT (mutant) stable cell lines were plated on 20 mm plates on the first day for attachment. On the second day, DMSO, BMX-IN-1, or BMX-IN-1R (10 μM) was added to each well for 4 h before washout and replaced with DMSO or 50 μg/mL of CHX for 16 or 24

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h. Cells were then washed in PBS and lysed in 200 μL of M-PER (Pierce) buffer supplemented with protease inhibitors and phosphatase inhibitor. The lysis procedure was performed according to the manufacturer's instructions. Mouse anti-BMX antibody (BD Bioscience) and mouse anti-tubulin (Sigma) were used for immunoblotting. Quantification was done using image J software.

Flow Cytometry Cell Cycle and Apoptosis Analysis. RV-1 cells in complete or serum-reduced DMEM were treated with DMSO, BMX-IN-1 (2.5 μM), MK2206 (200 nM), or the combination of BMX-IN-1 and MK2206 for 5 days before cells were harvested by trypsin and washed with cold PBS. The cells were then fixed in 70% cold ethanol (prechilled at −20 °C) and incubated at 4 °C overnight. On the day of flow cytometry, cells were collected by centrifugation, washed with PBS, and stained in 50 μg/mL propidium iodide (Sigma) + 0.5 mg mL^{-1} RNase (Sigma) in PBS + 0.5% Triton-X100 for 30 min at RT and moved to 4 °C until the time of analysis. Flow cytometry was performed using a BD FACScan, and results were analyzed by ModFit software in the Flow Cytometry Core Facility in Dana-Faber Cancer Institute.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Supplementary experimental procedures, supplementary Table 1, supplementary Figures 1−10, and supplementary references. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

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Notes

The authors declare no competing financial interest.

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