A Novel Small-Molecule Inhibitor of Mcl-1 Blocks Pancreatic Cancer Growth In Vitro and In Vivo

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Introduction

Pancreatic cancer is among the most therapy resistant cancers with poor prognosis and high mortality rate (1). Primary or acquired resistance of pancreatic cancer to chemotherapy and radiation therapy as a consequence of apoptosis defects is a major cause of treatment failure and poor outcome (2, 3). Hence, future efforts toward development of novel therapies to improve survival and quality of life of patients with pancreatic cancer should include new strategies that specifically target resistance of cancer cells to apoptosis (4).

Abstract

Using a high-throughput screening (HTS) approach, we have identified and validated several small-molecule Mcl-1 inhibitors (SMI). Here, we describe a novel selective Mcl-1 SMI inhibitor, 2 (UMI-77), developed by structure-based chemical modifications of the lead compound 1 (UMI-59). We have characterized the binding of UMI-77 to Mcl-1 by using complementary biochemical, biophysical, and computational methods and determined its antitumor activity against a panel of pancreatic cancer cells and an in vivo xenograft model. UMI-77 binds to the BH3-binding groove of Mcl-1 with \( K_i \) of 490 nmol/L, showing selectivity over other members of the antiapoptotic Bcl-2 family. UMI-77 inhibits cell growth and induces apoptosis in pancreatic cancer cells in a time- and dose-dependent manner, accompanied by cytochrome c release and caspase-3 activation. Comunoprecipitation experiments revealed that UMI-77 blocks the heterodimerization of Mcl-1/Bax and Mcl-1/Bak in cells, thus antagonizing the Mcl-1 function. The Bax/Bak-dependent induction of apoptosis was further confirmed using murine embryonic fibroblasts that are Bax- and Bak-deficient. In an in vivo BxPC-3 xenograft model, UMI-77 effectively inhibited tumor growth. Western blot analysis in tumor remnants revealed enhancement of proapoptotic markers and significant decrease of survivin. Collectively, these promising findings show the therapeutic potential of Mcl-1 inhibitors against pancreatic cancer and warrant further preclinical investigations.

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Myeloid cell leukemia-1 (Mcl-1) is a potent antiapoptotic protein, a member of the prosurvival Bcl-2 family, and its role is emerging as a critical survival factor in a broad range of human cancers including pancreatic cancer (5, 6). Functional studies have confirmed that Mcl-1 is capable of blocking apoptosis induced by various apoptotic stimuli including chemotherapy and radiation (7). Mcl-1 is highly expressed at the protein level in pancreatic cancer cells and is associated with resistance to chemotherapeutic agents (8–13). It has been shown that downregulation of Mcl-1 enhances the induction of apoptosis and sensitivity of pancreatic cancer to gemcitabine and radiation (11, 12). Thus, Mcl-1 represents an attractive molecular target for development of a new class of cancer therapy for treatment of pancreatic cancer.

The most potent small-molecule inhibitors of the Bcl-2 subfamily described to date are the Bad-like BH3 mimetics (14–16). ABT-737, one of these mimetics, binds with high affinity (\( K_i \leq 1 \) nmol/L) to Bcl-2, Bcl-xL, and Bcl-w but fails to bind to Mcl-1 (14). Studies have shown that resistance to ABT-737 is linked to high expression levels of Mcl-1 and in many instances this resistance can be overcome by treatment with agents that downregulate, destabilize, or inactivate Mcl-1 (17, 18). It was shown that knockdown of Mcl-1 sensitizes human pancreatic cancer cells to ABT-737–induced apoptosis, indicating that Mcl-1 is a relevant therapeutic target in these cancer cells (13).
Using a high-throughput screening (HTS) approach, we have identified and validated several small-molecule Mcl-1 inhibitors. Here, we describe a novel selective small-molecule Mcl-1 inhibitor, 2 (UMI-77), an analog of the lead compound 1 (UMI-59), that selectively binds Mcl-1, induces Bax-Bak dependent apoptosis in pancreatic cancer cells, and exhibits single-agent antitumor activity in a BxPC-3 xenograft model, providing promising evidence for therapeutic potential of Mcl-1 inhibitors against pancreatic cancer.

Materials and Methods

Chemistry information

Synthesis and characterization of compounds 1 (UMI-59) and 2 (UMI-77) are provided in the Supplementary Methods. Compound 3 (UMI-101) was purchased from Princeton BioMolecular Research.

Protein purification

Five recombinant antiapoptotic Bcl-2 proteins were used in the binding studies: Mcl-1, Bcl-2, Bcl-xL, Bcl-w, and A1/Bfl-1 (for details see Supplementary Methods).

Fluorescence polarization–based binding assays

IC50 and Ki values of Mcl-1 inhibitors to antiapoptotic proteins from Bcl-2 family were determined in FP-based competitive binding assays provided in the Supplementary Methods. The Ki values were calculated as described previously (19).

Surface plasmon resonance binding assays

SPR experiments were carried out on Biacore 2000 optical biosensor using immobilized recombinant Bax (Novus Biologicals) and biotin-labeled Bid BH3 peptide in competitive solution binding experiments (for details see Supplementary Methods).

Induced fit docking

Crystal structure of Mcl-1 with mouse Noxa BH3 peptide (PDB ID: 2NLA) was used to model the binding pose of 2 (UMI-77) (ref. 20). The Schrödinger’s induced fit docking (IFD) protocol (21), provided in the Supplementary Methods, was used for the docking studies, and the docking pose was refined with molecular dynamic simulation program of Schrödinger’s MacroModel (22).

Biotin streptavidin pull-down experiment

Human breast cancer 2LMP cells, a subclone of the MDA-MB-231 cell line, were lysed in CHAPS buffer (10 mmol/L HEPES (pH 7.4), 2.5 mmol/L EDTA, 150 mmol/L NaCl, 1.0% CHAP). Pre cleared cell lysates were incubated with different concentrations of compounds followed by incubation with biotinylated Noxa BH3 peptide (18–43) and streptavidin-agarose beads to pull-down Mcl-1 protein bound to Noxa peptide. Beads were washed with CHAPS buffer, and Mcl-1 protein was eluted by boiling in SDS-PAGE sample buffer and analyzed by Western blotting using Mcl-1 antibody (Santa Cruz).

Cells and growth inhibition

All pancreatic cancer cell lines were obtained from American Type Culture Collection (ATCC). The cells have been tested and authenticated in the Applied Genomics Technology Center at Wayne State University in March 2011. The method used for testing was short tandem repeat (STR) profiling using the PowerPlex(r) 16 System from Promega. Human pancreatic cancer cell lines AsPC-1, BxPC-3, and Capan-2 were cultured in RPMI-1640 medium, whereas Panc-1 and MiaPaCa were cultured in Dulbecos’ Modified Eagle’s Medium (DMEM; Life Technologies), all supplemented with 10% FBS (Thermo Scientific HyClone). The cell growth inhibition after treatment with increasing concentrations of the compounds was determined by WST-8 assay (Dojindo Molecular Technologies Inc.).

Quantification of apoptosis

An Annexin-V-FLUOS/Propidium iodide staining kit and ELISA kit (Roche Applied Science) were used to detect apoptosis in pancreatic cancer cells. Cells were treated with Mcl-1 inhibitors for different time points, harvested, washed with PBS and apoptosis was quantified according to manufacturer’s protocol.

Immunofluorescence microscopy

Cells (3 x 10^3 to 4 x 10^5) were seeded on glass coverslips in 6-well cell culture dishes, allowed to attach overnight, and treated with UMI-77 for 24 hours. Cells were washed with PBS, a secondary antibody labeled with DyLight 488 (Thermo Scientific) was added and incubated for 2 hours at room temperature. Nuclei were visualized with 4,6-diamidino-2-phenylindole (DAPI). Samples were analyzed with a FluoView 500 Confocal Laser Scanning Microscope (Olympus). The fluorescent intensity of the active Bax was quantified using image processing program ImageJ 1.47 (NIH).

RNA interference

Human pancreatic cancer cells were transfected with Mcl-1 siRNA and control siRNA, respectively (both from Santa Cruz), using Lipofectamine 2000 as described in the manufacturers protocol (Cell Signaling).

Western blot analysis

The cells were treated and harvested at the indicated time points. Total cell lysates were subjected to electrophoresis, transferred to polyvinylidene difluoride (PVDF) membranes, and incubated with a specific primary antibody, followed by visualization with chemiluminescent detection reagent (Roche Molecular Biochemicals). Primary antibodies included: caspase-3 (Enzo Life Sciences); Mcl-1 and actin (Santa Cruz); Bcl-xL (BD Transduction Laboratories).

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Mcl-1 Inhibitors as Potential Therapeutics for Pancreatic Cancer

Loratories); Bcl-2, Bax, pro-PARP, and cytochrome c (Cell Signaling); Bak (Calbiochem); and Smac (Abgent).

Immunoprecipitation

Cell lysate (500 μg) was subjected to immunoprecipitation by adding 2.5 to 5 μg of anti-Mcl-1 antibody and incubation overnight at 4°C. After adding 30 μL of Protein G-agarose (Immunoprecipitation Kit, Sigma) and incubation for 4 hours, the samples were centrifuged. The agarose pellet was washed, resuspended in Laemmli buffer (Santa Cruz), boiled and supernatant was used for Western blot analysis.

Metabolic stability assay

Metabolic stability of UMI-77 was determined using the pooled mice liver microsomes (XenoTech, LLC). The conditions of the assay and quantification of UMI-77 in different time points are provided in Supplementary Information.

Animal preclinical efficacy trail design

For BxPC-3 subcutaneous model, 10 × 10⁶ cells were subcutaneously injected into the flanks of 4- to 5-week-old female severe combined immunodeficient mice (ICR-SCID, Taconic Farms). Palpable tumors started to appear in 3 to 5 weeks (23). Tumors were measured twice weekly. To prevent any pain or discomfort, mice were euthanized when their tumors reached 3 to 5 cm². Tumors were measured twice weekly. At the end of the treatment period, animals were euthanized and their tumors harvested for protein isolation and Western blot analysis for apoptotic markers.

Statistical analysis

Statistics was evaluated using GraphPad StatMate software (GraphPad Software, Inc.). P < 0.05 or P < 0.01 was used to indicate statistical significance.

Results

Compound 2 (UMI-77) selectively binds Mcl-1

Applying a HTS approach, we have screened a library of 53,000 synthetic small molecules available at the Center for Chemical Genomics, University of Michigan using a fluorescence polarization (FP)-based binding assay. Compound 1 (UMI-59, Fig. 1A) is one of the validated hits, which was resynthesized and confirmed its binding to Mcl-1 protein (Supplementary Scheme 1). In this article, we report compound 2 (UMI-77), an analog of the lead compound UMI-59 with improved binding affinity to Mcl-1.

The binding affinity and selectivity of 2 (UMI-77) against 5 members of Bcl-2 family of proteins was determined using FP-based binding assays (Fig. 1B and Table 1). The obtained results showed that UMI-77 selectively and potently displaced fluorescent-labeled BID-BH3 peptide from Mcl-1 protein with a Ki of 0.49 μmol/L, showing 3 times higher potency for binding to Mcl-1 than 1 (UMI-59; Ki = 1.55 μmol/L). Compound 3 (UMI-101), an analog of UMI-59, did not show binding to Mcl-1 up to 100 μmol/L and therefore was used as a negative control in cell-based assays. The binding profile studies showed that UMI-77 displayed significantly decreased binding affinities to the rest of the antiapoptotic proteins and its binding selectivity was consistent with the structural similarities between Mcl-1 and the antiapoptotic members of Bcl-2 family. UMI-77 bound to A1/Bfl-1 with 11-fold lower affinity than to Mcl-1 (Ki = 5.33 μmol/L), followed by Bcl-w with Ki = 8.19 μmol/L (17-fold decreased), and more than 50-fold reduced binding to Bcl-2 (Ki = 23.83 μmol/L) and Bcl-xL (Ki = 32.99 μmol/L). To confirm the binding selectivity of UMI-77 to antiapoptotic proteins, a solution competitive surface plasmon resonance (SPR)-based binding assay using a biotin-labeled Bid peptide immobilized on streptavidin chip was conducted. Consistent with FP results, UMI-77 showed 4-fold improved binding affinity to Mcl-1 (IC50 = 0.31 μmol/L) in comparison with UMI-59 (IC50 = 1.23 μmol/L; Supplementary Fig. S1A) and more than 30-fold decreased binding to Bcl-2 and Bcl-xL (Supplementary Fig. S1B).

To extend these findings to a cellular context, we used a pull-down assay using a biotin-labeled Noxa BH3 peptide (BL-Noxa) to probe whether UMI-77 interacts with cellular Mcl-1 protein. BL-Noxa selectively pulls down cellular Mcl-1 from 2LMP cell lysate and both compounds, UMI-59 and UMI-77, effectively disrupts the interactions between BL-Noxa and cellular Mcl-1 in a dose-dependent manner (Fig. 1C). Consistent with our binding results, UMI-77 is more potent than UMI-59 and blocks this interaction starting from 10 μmol/L. These data show that UMI-77 binds the endogenous, cellular Mcl-1 protein and blocks the binding of BL-Noxa to Mcl-1.

It was reported that Mcl-1 regulates proapoptotic Bax and Bak proteins through binding to their BH3 domains and preventing their proapoptotic activity (24, 25). Accordingly, we have developed an SPR-based binding assay to test the ability of Mcl-1 inhibitors to block the Mcl-1/Bax protein–protein interactions. Recombinant Bax protein (residues 1–100) was immobilized and it was determined that Mcl-1 binds to Bax with a Ki value of 217 nmol/L (Supplementary Fig. S2). Preincubation of the Mcl-1 protein with increasing concentrations of UMI-77 blocked the binding of Mcl-1 to Bax in a dose-dependent manner with an IC50 of 1.43 μmol/L (Fig. 1D). Noxa BH3 peptide inhibits the binding of Mcl-1 to Bax with an IC50 of 0.062 μmol/L, 23-fold more potent than UMI-77. Consistent with the results obtained from the FP-based assay, UMI-101 failed to disrupt the Mcl-1/Bax protein–protein interactions. These results confirmed that UMI-77 binds to the Mcl-1 protein and is capable of disrupting Mcl-1/Bax protein–protein interactions.
2 (UMI-77) binds to the BH3-binding pocket of Mcl-1 protein

To confirm the binding of 2 to the BH3 groove of Mcl-1 protein and to explore the interaction between the UMI-77 and Mcl-1, in silico docking analysis and heteronuclear single and quantum correlation (HSQC) NMR spectroscopy studies were conducted. The interactions between helical BH3 domain of proapoptotic and the BH3-binding groove in antiapoptotic proteins are well-characterized (Supplementary Fig. S3). They involve hydrophobic interactions through 4 conserved hydrophobic residues of the BH3 domain in proapoptotic proteins and a salt bridge between conserved aspartic acid and arginine on the antiapoptotic proteins. Mimicking these interactions is the main strategy toward developing small-molecule BH3 mimetic Mcl-1 inhibitors (26).

The predicted binding model of UMI-77 in the complex with Mcl-1 revealed that UMI-77 occupies 2 hydrophobic pockets in Mcl-1, h2 and h3, mimicking 2 conserved hydrophobic residues from mNoxaB (PDB ID:2NLA), Leu78, and Ile81, respectively (Fig. 2A and Supplementary Fig. S3). Specifically, the p-bromophenyl group inserts into the h2 pocket and has hydrophobic interactions with Met231, Met250, Val253, Leu267, and Phe270. The proposed interactions were confirmed with the HSQC NMR experiments in which these residues showed significant chemical shift changes.

### Table 1. Binding affinities of 1 (UMI-59) and 2 (UMI-77) to five antiapoptotic Bcl-2 family members in competitive FP assay using fluorescent-labeled Bid BH3 peptide

<table>
<thead>
<tr>
<th>Protein</th>
<th>1 (UMI-59)</th>
<th>2 (UMI-77)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mcl-1</td>
<td>1.55 ± 0.18</td>
<td>0.49 ± 0.06</td>
</tr>
<tr>
<td>A1/Bfl-1</td>
<td>6.14 ± 1.0</td>
<td>5.33 ± 1.0</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>54.65 ± 9.56</td>
<td>23.83 ± 1.81</td>
</tr>
<tr>
<td>Bcl-xL</td>
<td>99.0 ± 22.63</td>
<td>32.99 ± 4.33</td>
</tr>
<tr>
<td>Bcl-w</td>
<td>37.53 ± 7.96</td>
<td>8.19 ± 1.91</td>
</tr>
</tbody>
</table>
significant chemical shift perturbations (>60 Hz; Fig. 2B and D). The naphthalene ring of UMI-77 occupies the h3 pocket and makes hydrophobic interactions with Phe228, whereas the 4-hydroxyl group forms a hydrogen bond with His224. This residue was identified as an acidic hotspot in the h3 site of Mcl-1 (27), supporting our predicted electrostatic interaction in this region of Mcl-1 (Fig. 2A). Consistent with the computational model, NMR experiments showed that Met231 and Val220 from the h3 pocket, have significant chemical shift perturbations (>60 Hz), as well as His224 and Phe228 (between 30 and 60 Hz), confirming our predicted interactions of the UMI-77 in this region of the Mcl-1 protein (Fig. 2B and D). The docking model revealed that the carboxyl group of UMI-77 forms hydrogen bonding network with Arg263 and Asn260, mimicking the conserved aspartate in proapoptotic proteins. Indeed, the HCQ5 NMR spectrum of UMI-77/Mcl-1 complex showed that Arg263 has a significant chemical shift (Fig. 2B-D). Overall analysis of the chemical shifts of the Mcl-1/UMI-77 and Mcl-1/Bim BH3 peptide complexes showed that UMI-77 affected the same residues as Bim BH3 peptide (Supplementary Fig. S4). Taken together, in silico docking and HSQC NMR studies provided conclusive evidence that UMI-77 binds to the BH3-binding groove of Mcl-1 protein. To understand the selective binding of UMI-77 to Mcl-1, we compared its binding model to the reported selective Mcl-1 SMI, maritoclax (28), as well as with the Bims2A, a selective Mcl-1 BH3-like peptide derived from Bim peptide (29). Interestingly, the two SMIs have different binding modes, UMI-77 occupies the h2 and h3 hydrophobic pockets, whereas maritoclax binds near the h4 hydrophobic pocket. However, structural studies of Bims2A showed that the hydrophobic residue in position h3, Ile65 (Supplementary Fig. S3) is the most critical for the selective and high affinity binding to Mcl-1 (29). Consistent with this study, the naphthalene ring of UMI-77 occupies h3 pocket and mimics this hydrophobic residue. During the revision of this article, the first complex structure between small-molecule inhibitor and Mcl-1 was reported (30) confirming the importance of the h2 and h3 hydrophobic pockets for selective targeting of Mcl-1 and providing insights for understanding the structural determinants for selective targeting of Mcl-1 protein.
2 (UMI-77) inhibits growth of pancreatic cancer cells and induces intrinsic apoptotic pathway

Cytoxic effect of 2 was evaluated using a panel of 5 pancreatic cancer cell lines with different expression level of anti- and proapoptotic proteins (Supplementary Fig. S5). A dose–response analysis revealed that UMI-77 most potently inhibits the cell growth of BxPC-3 and Panc-1 cell lines with IC_{50} values of 3.4 and 4.4 μmol/L, respectively, and shows 3 to 5 times less potency in inhibition of the cell growth of 2 other tested cell lines MiaPaCa-2 (12.5 μmol/L) and AsPC-1 (16.1 μmol/L; Fig. 3A). The cell growth inhibition potency of UMI-77 correlates with the highest expression of Mcl-1 and Bak and lowest expression of Bcl-xL in the sensitive cell lines, BxPC-3 and Panc-1. Consistent with this, less sensitive pancreatic cancer cells, MiaPaCa and AsPC-1, express low level of Mcl-1 and Bak and high expression of Bcl-xL which may contribute to their less sensitivity as UMI-77 selectively binds Mcl-1 and shows more than 50-fold reduced binding to Bcl-xL. Interestingly, Capan-2 cells are showing similar sensitivity to UMI-77 (IC_{50} = 5.5 μmol/L) as BxPC-3 and Panc-1, although they have low Mcl-1 levels. One of the potential explanations might be the p53 status as only Capan-2 cells express wild type p53, whereas all other tested pancreatic cancer cell lines express mutated p53. Differences in p53 status might influence the efficacy of UMI-77 in Capan-2 cell line as it is known that p53 activates the transcription of Puma and Noxa, thus shifting the balance of the Bcl-2 family toward proapoptotic members (31–33). p53-mediated induction of Puma might enhance the sensitivity of Capan-2 cells by lowering the threshold set by Bcl-xL, which is highly expressed in these cells. However, further mechanistic studies are needed, which is beyond the scope of this article. Importantly, the specificity of cell growth inhibition by UMI-77 was confirmed by experiments with compound 3 which showed no binding to Mcl-1 at concentrations up to 100 μmol/L and failed to show inhibition of the cell growth in all tested pancreatic cancer cells.

To gain insights into the underlying mechanism of action for the cell growth inhibition of UMI-77, we selected BxPC-3 and Panc-1 cell lines for further investigation. To determine whether apoptosis contributes to the anti-proliferative effect of UMI-77, BxPC-3 and Panc-1 cells were treated with increasing concentrations of this compound for different times. Induction of apoptosis was monitored by flow cytometry using Annexin V and propidium iodide double staining. UMI-77 was effective in induction of apoptosis in a time- and dose-dependent manner in Panc-1 cells (Fig. 3B). Treatment of the Panc-1 cells with 5 and 10 μmol/L concentrations of UMI-77 resulted in 15% and 21%, respectively, of early apoptotic cells after 24-hour treatment, and 21% and 49% after 48-hour treatment. Similar results were also obtained in BxPC-3 cells (Supplementary Fig. S6). The inactive compound 3 even at concentration of 100 μmol/L failed to induce apoptosis in both tested pancreatic cancer cell lines. AsPC-1 cells which are less sensitive to UMI-77, as was expected, showed minimum induction of early apoptosis (8%) in the highest tested concentration (20 μmol/L; Supplementary Fig. S6). Next, we examined whether UMI-77 could directly induce apoptosis through activation of the intrinsic mitochondrial pathway. As cytochrome c release from mitochondria to the cytosol and subsequent activation of caspases represent key steps during intrinsic apoptosis, we first determined whether UMI-77 could affect this process in Panc-1 cells. Treatment for 24 hours of Panc-1 cells with UMI-77 resulted in dose-dependent release of cytochrome c and Smac from mitochondria, starting at a concentration of 10 μmol/L (Fig. 3C). Similar results were obtained after treatment of BxPC-3 cells (Supplementary Fig. S7). The induction of apoptosis and release of cytochrome c was accompanied by PARP cleavage (Fig. 3D) and activation of caspase-3 quantified by densitometric analysis, where two concentrations of UMI-77 (3 and 10 μmol/L) showed statistically significant cleaved caspase-3 versus DMSO control (Fig. 3E). These results showed that UMI-77 induced apoptosis in pancreatic cancer through activation of the intrinsic apoptotic pathway.

In the indirect activation model (34), the activity of the apoptosis effectors Bak and Bax can be suppressed by multidomain antiapoptotic proteins such as Mcl-1. If cell death induction is specifically mediated by Mcl-1 protein, Bak or Bax would be required for release of cytochrome c and subsequent cell death. Therefore, to investigate whether UMI-77–induced apoptosis involved the disruption of Mcl-1/Bax and/or Mcl-1/Bak complexes in pancreatic cancer, immunoprecipitation studies were conducted. Treatment of BxPC-3 cells with UMI-77 at 4 μmol/L for 24 hours resulted in inhibition of the endogenous protein–protein interactions of Bax and Bak with Mcl-1, indicating that UMI-77 induced cell death and apoptosis is through attenuating the ability of Mcl-1 to sequester proapoptotic proteins such as Bax and Bak (Fig. 3F). To further elucidate the role of Bak and Bax, wild-type (WT) murine embryonic fibroblasts (MEF) and double knockout (DKO) cells, deficient in both Bax and Bak, were used. UMI-77 at a concentration of 10 μmol/L induced more than 60% apoptosis in the MEF WT cells, whereas at the same concentration, the induction of apoptosis in MEF DKO cells was significantly reduced showing only 16% apoptotic cells (Fig. 3G). These results showed that UMI-77 induced apoptosis in a Bax/Bak-dependent manner. To further interrogate the mechanism of action, we examined the activation of Bax in Panc-1 and BxPC-3 cells after treatment with UMI-77 using the 6A7 anti-Bax antibody, which specifically recognizes the conformationally active form of Bax (35). The quantification of the fluorescent intensity of the active Bax in the cells treated with UMI-77 showed a substantial increase in activated Bax in both tested pancreatic cell lines starting from 5 μmol/L (Fig. 3H and 3I and Supplementary Fig. S8). These data suggest that UMI-77 treatment leads to Bax conformational change, consistent with our coimmunoprecipitation and functional studies.
Figure 3. 2 (UMI-77) effect on pancreatic cancer cell growth and induction of apoptosis. A, IC50 values of cell growth inhibition of 2 (UMI-77) in panel of pancreatic cancer cell lines after 4-day treatment. B, time- and dose-dependent induction of apoptosis in Panc-1 cells after treatment with 2 (UMI-77). Cells were treated for different time points, and apoptosis was determined with Annexin V/PI double staining. C, release of cytochrome c and Smac from mitochondria in Panc-1 cells. Cells were treated for 24 hours, mitochondria were isolated, and cytochrome c and Smac were probed by Western blotting. D, full-length PARP (FL-PARP), cleaved PARP, and activation of caspase-3 in BxPC-3 cells after 24-hour treatment with 2 (UMI-77). Cells were treated for 24 hours, and caspase-3 and PARP were probed by Western blotting. E, cleaved caspase-3 was quantified by densitometric analysis and presented in graphical form. The statistical significance was calculated with minimum of three values for all tested concentrations (n = 3; *, P < 0.05). F, immunoprecipitation on 2 (UMI-77)-treated BxPC-3 cell lysate was conducted using Mcl-1 antibody followed by Western blot analysis with Bax or Bak. G, induction of apoptosis in wild-type (WT) and Bax/Bak-deficient (DKO) MEFs cells after 24-hour treatment with 10 μmol/L of 2 (UMI-77). H and I, 2 (UMI-77) induces Bax activation in Panc-1 cells. Immunocytochemistry analysis (H) and quantification of the fluorescence intensity (I) showed the increased number of positive cells stained with anti-Bax(6A7) antibody, which specifically detects the active form of Bax, 24 hours after 2 (UMI-77) treatment in Panc-1 cells. Conversely, dimethyl sulfoxide (DMSO) control did not induce the active form of Bax [green, anti-Bax(6A7) antibody; blue, DAPI]. Scale bar, 100 μm. Forty cells were used for quantification of the fluorescence intensity. All experiments were carried out minimum 3 times and the most representative results are presented.
Knocking down Mcl-1 expression abrogates growth inhibition and apoptosis by UMI-77

To further confirm the functional role of Mcl-1 in UMI-77–mediated cell growth inhibition and induction of apoptosis, we examined whether siRNA-mediated silencing of Mcl-1 expression would affect UMI-77 induced apoptosis. Cells were treated for 72 hours with the 3.5 μmol/L of UMI-77, after 24 hours of siRNA transfection. UMI-77 induced 50% cell growth inhibition and 7-fold induction of apoptosis in BxPC-3 cells transfected with control siRNA (Fig. 4A and B). However, when BxPC-3 cells were treated with UMI-77 in the presence of Mcl-1 siRNA, sensitivity of BxPC-3 cells to UMI-77–induced cell growth and apoptosis were significantly reduced by Mcl-1 silencing (P < 0.05) in comparison with cells transfected with control siRNA. In addition, the level of induced apoptosis in BxPC-3 cells treated with UMI-77 and Mcl-1 siRNA was similar as in the cells treated only with Mcl-1 siRNA, clearly suggesting that UMI-77–induced apoptosis is mediated by Mcl-1 (Fig. 4B). Immunoblot analysis indicated that Mcl-1 siRNA completely and specifically inhibits Mcl-1 protein expression under the treatment condition and not in control siRNA samples, confirming efficient silencing (Fig. 4C). Collectively, these data are emphasizing the critical role of Mcl-1 in UMI-77 cellular efficacy in pancreatic cancer.

UMI-77 exhibits single-agent antitumor activity in BxPC-3 xenograft model

The in vitro data described earlier prompted us to extend these observations and test the in vivo efficacy of UMI-77 in a BxPC-3 xenograft model in SCID mice. First, we tested its in vitro microsomal stability by incubating UMI-77 with pooled mice liver microsomes. UMI-77 exhibited moderate metabolic stability with a half-life of 45 minutes (Fig. 5A). This result was promising for continuation with in vivo efficacy studies in view of the fact that in vitro microsomal stability correlates with the in vivo plasma clearance. Second, we determined the maximum tolerated dose (MTD) of UMI-77 in SCID mice. Administered 60 mg/kg i.v. for 5 consecutive days per week for 2 weeks did not cause any loss in the animal weight, and there was no obvious sign of toxicity during the course of the treatment (Fig. 5B). Increasing the dose to 80 mg/kg showed severe animal weight loss (>20%), therefore 60 mg/kg was used as a therapeutic dose for the in vivo efficacy studies. Daily treatment with UMI-77 for 5 consecutive days a week for 2 weeks resulted in statistically significant tumor growth inhibition by 65% and 56% in comparison with the controls in day 19 (P < 0.0001) and day 22 (P < 0.003), respectively (Fig. 5C). To elucidate the molecular mechanism of UMI-77–mediated tumor growth inhibition, Western blotting and immunohistochemistry on tumor tissue were conducted. The Western blot analyses of the tumor tissue lysates showed slightly elevated levels of proapoptotic proteins, Bax and Bak, and significant decrease of survivin, one of the inhibitors of apoptosis proteins (IAP) which potently inhibits apoptosis by antagonizing caspase activity (Fig. 5D). The apoptotic cells in tumor tissue were determined by TUNEL-based in situ method, and the obtained results showed that positive apoptotic cells of tumor sections were significantly increased in UMI-77-treated BxPC-3 xenograft mice as compared with the control group (Fig. 5E). The toxicity of UMI-77 on normal tissues was examined by hematoxylin and eosin (H&E) analyses (Supplementary Fig. S9). Histopathology revealed that treatment of mice with UMI-77 did not cause damage to tested tissues from kidney, liver, and pancreas, showing that it is not toxic to normal mouse tissues. These findings provide in vivo support of the involvement of Mcl-1 regulated pathway in pancreatic cancer, implicating the potential of Mcl-1 inhibitors as novel antitumor agents for treatment of pancreatic cancer.

Figure 4. Downregulation of Mcl-1 by siRNA in BxPC-3 cells blocks growth inhibition and apoptosis induced by 2 (UMI-77). A, cell growth inhibition determined with MTT assay. B, induction of apoptosis detected using Histone/DNA ELISA assay. C, Mcl-1 protein expression in nontransfected Bx-PC3 cells and transfected with control siRNA and Mcl-1 siRNA. Cells were transiently transfected with siRNA against Mcl-1 or control siRNA for 24 hours and then treated with the indicated concentration of UMI-77 for 72 hours. **, P < 0.05 versus control siRNA and UMI-77 3.5 μmol/L. The experiment was replicated 2 times independently and gave consistent results.
The present study is the first report showing the in vitro and in vivo efficacy of novel, selective small-molecule Mcl-1 inhibitor 2 (UMI-77) in pancreatic cancer. A hallmark of cancer cells is defects in the apoptotic cell death program (36), and research on antiapoptotic resistance mechanisms has shown that Mcl-1 represents an important survival factor for pancreatic cancer (8–10). Therefore, the obtained results provide significant proof of concept that SMIs that can bind Mcl-1 and block its interactions with proapoptotic proteins may have potential as a new targeted treatment for pancreatic cancer by overcoming the apoptosis resistance mechanism.

Mcl-1 is a homologous protein related to other antiapoptotic proteins such as Bcl-2 and Bcl-xL, but there are subtle differences in the hydrophobic binding groove in Mcl-1 that translate into selective binding to the proapoptotic BH3-only proteins (37, 38). Using HTS, we have identified and validated a novel, selective Mcl-1 inhibitor, 1 (UMI-59) that binds to the BH3-binding groove and antagonizes its function. Chemical modifications of this compound led to 2 (UMI-77) with improved binding affinity to Mcl-1.

**Discussion**

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In vitro FP-based binding studies showed that UMI-77 selectively binds to Mcl-1 with $K_i$ of 490 nmol/L and has significantly decreased binding affinity to the additional 4 members of antiapoptotic proteins. Docking and H5QC NMR binding studies provide conclusive evidence that UMI-77 binds to the BH3-binding groove of Mcl-1 protein and the interactions are mediated by 2 conserved hydrophobic pockets, h2 and h3, and the hydrogen bonding network including the conserved hydrogen bond interaction with Arg263.

**Figure 5.** In vivo characterization of 2 (UMI-77). A, determination of the microsome stability of UMI-77 (expressed as $T_{1/2}$). B, evaluation of the effect of UMI-77 on weight loss of SCID mice. UMI-77 was administrated 2 cycles i.v. for 5 d/wk in 2 tested concentrations 60 and 80 mg/kg. C, in vivo efficacy of UMI-77 in BxPC-3 xenograft animal model. BxPC-3 xenografts were inoculated subcutaneously in SCID mice. Once transplanted, fragments developed into palpable tumors, about 60 mg, groups of 4 animals with bilateral tumors were removed randomly and assigned to 2 treatment groups. Mice were administred UMI-77 i.v 60 mg/kg for 5 consecutive days a week for 2 weeks. Vehicle-treated group and UMI-77–treated groups showed significant reduction of the tumor growth when compared to treatments with vehicle on days 19 ($^* P < 0.0001$), 21 ($^* P < 0.003$), and 22 ($^* P < 0.003$). D, Western blot analysis for different proapoptotic and survival markers on lysates isolated from tumors harvested from mice of different treatment groups showing enhancement of proapoptotic Bax and Bak and downregulation of survivin compared with control. E, Immunohistochemical staining of BxPC-3 tumor xenografts using Apoptag Kit. No positively staining nuclei are present in the control samples. Several positively staining cells (open arrows) are present, as is apoptotic debris (closed arrows) in presented field (400× original magnification).
From a functional standpoint, UMI-77 effectively targets endogenous Mcl-1 and induces apoptosis in a time- and dose-dependent manner. Apoptosis induction occurs at low micromolar doses correlating well with the in vitro binding affinity of this compound to Mcl-1 as well as its potency in inhibition of the pancreatic cancer cell growth. Mechanistically, apoptosis induction by UMI-77 is Bax/Bak-dependent (Fig. 3G), preceded by disrupting the Mcl-1/Bak and Mcl-1/Bax complexes (Fig. 1D and 3F), followed by Bax activation (Fig. 3H and 3I), which results to a mitochondria-mediated cell death (Fig. 3C and D; refs. 39–41). These results suggest that UMI-77 functions as BH3 mimetic and exhibits specific and mechanism-based cell growth inhibition. Using siRNA interference approach, knocking down the Mcl-1 expression significantly decreased induction of apoptosis and protected pancreatic cancer cells from killing induced by UMI-77 in comparison with control siRNA–transfected cells (Fig. 4), showing that Mcl-1 is a mediator of cell sensitivity to this compound and the effect is Mcl-1 dependent.

UMI-77 showed robust antitumor efficacy in a resistant pancreatic cancer xenograft model with no toxicity to the surrounding tissue and minimal discomfort to the host. Molecular analysis of tumor remnants showed a significant decrease of the antiapoptotic protein survivin, which potently inhibits apoptosis through antagonizing caspase activity. In situ apoptosis detection assays confirmed that UMI-77 induces apoptosis in the tumor tissue but not in the control treated tumors.

In summary, we have shown that UMI-77, a novel selective small-molecule Mcl-1 inhibitor, potently inhibits pancreatic cancer growth in vitro and in vivo. Our results provide insights into the potential role of Mcl-1 as a therapeutic target in pancreatic cancer treatment and suggest that antagonizing Mcl-1 function with small molecules as UMI-77 warrants further investigation as a therapeutic strategy against pancreatic cancer. In particular, future studies are necessary for testing UMI-77 in combination with chemotherapy and radiotherapy for the development of new therapeutic strategies to overcome the intrinsic and acquired resistance of pancreatic cancer cells and to enhance the treatment efficacy.

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

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