Structure-Function Analysis of the Mcl-1 Protein Identifies a Novel Senescence-regulating Domain*

Received for publication, May 6, 2015, and in revised form, July 21, 2015 Published, JBC Papers in Press, July 23, 2015, DOI 10.1074/jbc.M115.663898

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Background: Mcl-1 is a prosurvival gene critical for chemotherapy resistance. However, its antisenescent properties are poorly characterized.

Results: Through mutagenesis of Mcl-1 in functional assays, we identified a loop domain required for inhibiting senescence.

Conclusion: An internal loop domain of Mcl-1 is responsible for antisenescent functions.

Significance: Our study provides additional targets within Mcl-1 that can enhance senescence-inducing cancer treatments.

Unlike other antiapoptotic Bcl-2 family members, Mcl-1 also mediates resistance to cancer therapy by uniquely inhibiting chemotherapy-induced senescence (CIS). In general, Bcl-2 family members regulate apoptosis at the level of the mitochondria through a common prosurvival binding groove. Through mutagenesis, we determined that Mcl-1 can inhibit CIS even in the absence of its apoptotically important mitochondrion-localizing domains. This finding prompted us to generate a series of Mcl-1 deletion mutants from both the N and C termini of the protein, including one that contained a deletion of all of the Bcl-2 homology domains, none of which impacted anti-CIS capabilities. Through subsequent structure-function analyses of Mcl-1, we identified a previously uncharacterized loop domain responsible for the anti-CIS activity of Mcl-1. The importance of the loop domain was confirmed in multiple tumor types, two in vivo models of senescence, and by demonstrating that a peptide mimetic of the loop domain can effectively inhibit the anti-CIS function of Mcl-1. The results from our studies appear to be highly translatable because we discerned an inverse relationship between the expression of Mcl-1 and of various senescence markers in cancerous human tissues. In summary, our findings regarding the unique structural properties of Mcl-1 provide new approaches for targeted cancer therapy.

Overexpression of antiapoptotic molecules is a major mechanism by which tumors can continue to progress despite aggressive treatment with cytotoxic therapies. Among the molecules mediating these prosurvival effects are members of the Bcl-2 family, proteins now known to be key drivers of oncogenesis and growth in most human cancers. Large studies assessing somatic copy numbers of oncogenic proteins reveal that two members of the Bcl-2 family, Bcl-2 and Mcl-1, are particularly elevated in many forms of human cancer (1, 2). Characterizing these prosurvival proteins is a common canonical binding groove that sequesters and neutralizes proapoptotic proteins (3) and, therefore, is presumed to be essential for mediating tumor development and progression and resistance to therapeutic intervention (4). Consequently, continuous efforts are focused on developing inhibitors of this binding groove, with several candidate inhibitors already in clinical trials (5). Recent clinical trials reveal that inhibitors of prosurvival Bcl-2 family members that do or do not include targeting Mcl-1 can be effective in hematogenous cancers but are generally ineffective against solid tumors (6, 7).

Preclinical data reveal that solid tumors often survive selective inhibition of antiapoptotic Bcl-2 family members by up-regulating Mcl-1 (8), a protein whose unique binding groove is deemed responsible for its resilience and resistance to many of the drugs designed to inhibit the major Bcl-2 survival proteins, including Mcl-1 itself (9, 10). Therefore, there is a strong motivation to identify and/or design specific inhibitors of Mcl-1 that can abrogate its protective activity (11–15), perhaps by targeting a region of the molecule that differentiates it from other family members. Indeed, distinguishing Mcl-1 from other Bcl-2 family members is its appreciably larger, 350-residue size as well as its regulatory domain-containing N terminus, which modulates its function, localization, and stability/half-life (16–18). Because effectively targeting the anti-apoptotic binding groove of Mcl-1 continues to be a challenge, alternative strategies are being pursued, such as taking advantage of the particularly short half-life of the molecule (13, 19).

We have determined previously that Mcl-1 also possesses a unique ability to abrogate chemotherapy-induced senescence (CIS) both in vitro and in vivo (11). Surprisingly, untreated Mcl-1-depleted tumors xenografted into nude mice had significant impairment of tumor growth, not by an apoptotic mechanism but, instead, by undergoing a spontaneous form of senescence. Importantly, studies evaluating the effectiveness of cancer therapies provide compelling evidence that the level of

* The authors declare that they have no conflicts of interest with the contents of this article.

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2 The abbreviations used are: CIS, chemotherapy-induced senescence; CRC, colorectal cancer; BH, Bcl-2 homology.
Senescence correlates with overall clinical response, including prognosis (20). As a result, there are now a number of drugs in clinical cancer trials whose primary purpose is the induction of senescence (21). Therefore, on the basis of our work, there is a clinical need for a multifaceted approach to inhibit all aspects of Mcl-1 activity to both stimulate apoptosis and induce senescence in tumor cells.

In most cases, senescence signaling pathways are regulated by tumor suppressor genes such as retinoblastoma and p53 (22, 23). In our previous study, knockdown of endogenous Mcl-1 expression sensitized otherwise resistant cells to CIS, even in the absence of both p53 and retinoblastoma (11). Very recently, other groups have confirmed the unique role of Mcl-1 in promoting tumor progression in p53-deficient cancers (24). Therefore, Mcl-1 appears to be an additional mechanism of tumor senescence resistance above and beyond loss of tumor suppressor gene function.

This study focuses on the distinct structural aspects of Mcl-1 that impact its senescence regulation. Through extensive mutagenesis of Mcl-1 in functional assays, we eliminated all of the well known apoptosis-related structure of Mcl-1 as important in CIS regulation. Instead, we identified a so far unstudied loop domain as key to inhibiting CIS in vitro and in vivo as well as spontaneous senescence in vivo, thereby promoting tumor progression. The ramifications of this study are significant because current clinical efforts focus neither on the ability of Mcl-1 to regulate tumor senescence nor on this novel domain. As proof of principle for cancer therapy, we designed a cell-permeable peptide that is effective at inhibiting the endogenous anti-senescence activity of Mcl-1 via a dominant negative effect, sensitizing otherwise resistant cancer cells to CIS. We also show the strong linkage of Mcl-1 and senescence in human cancer tissue specimens, further highlighting the potential impact of targeting this protein. Overall, our study provides the framework for novel drug design to accelerate tumor cell senescence to increase the armamentarium of cancer therapies.

**Experimental Procedures**

**Cell Culture**—The human colorectal cancer (CRC) cell lines SW480, SW620, SW837, DLD-1, Caco2, RKO, HT-29, and HRT-18, a normal colon CRL-1459 cell line, and HeLa cells were obtained from the ATCC. HCT116 human colon cancer cell lines (p53+ and p53−/−) were provided by Bert Vogelstein (Johns Hopkins University). All cell lines were maintained in DMEM supplemented with penicillin/streptomycin, non-essential amino acids, and 10% FBS. The HN572 cell line was generated in our laboratory from surgically removed tumors and maintained in DMEM with supplements as described for other cell lines. The CCF2968 cell line was a gift from Dr. Jennifer Yu (Cleveland Clinic) and was derived in her laboratory. HCT116 p53−/− shcontrol, HCT116 p53−/− shMcl-1, and HCT116 shMcl-1 cells are derivatives of HCT116 p53−/− and HCT116, which stably express a transcript-specific shRNA that knocks down endogenous Mcl-1 expression or an irrelevant control RNA (Open Biosystems) and have been described previously (11). All cell cultures were incubated at 37 °C in a humidified incubator containing 5% CO₂.

**Site-directed Mutagenesis**—To assess relevant amino acid residues to the anti-senescence activity of Mcl-1, we used the QuikChange Lightning multisite-directed mutagenesis kit (Agilent Technologies/Stratagene) to convert each of those residues to an alanine (or to a glutamine) according to the protocol of the manufacturer. The resulting plasmids were sequenced to ensure that they encoded the appropriate amino acid substitution. Cells transiently transfected with the altered Mcl-1 containing vectors were then used in the indicated in vitro senescence assays.

**Plasmid Transfections and Drug Treatments**—Transient and stable plasmid transfection into the indicated cell lines was performed using Lipofectamine 2000 (Life Technologies) according to the instructions of the manufacturer. Briefly, 2 × 10⁵ cells/well in 6-well plates or 1 × 10⁵ cells/well in 6-well plates on poly-l-lysine-coated glass coverslips were transiently transfected with 0.5 μg of WT Mcl-1, various Mcl-1-expressing constructs, or empty pcDNA3.1 vector (Invitrogen). The medium was changed after 24 h, and then cells were incubated for 48 h prior to verifying transgene expression by Western blot analysis. Stable transfectants were selected with 600 μg/ml of genetin (Life Technologies) for 2 weeks. 48 h post-transfection, cells were left untreated or treated in fresh medium containing doxorubicin (100 ng/ml, Sigma) to induce senescence. Cell-permeable peptides of HIV TAT conjugated to a synthetic peptide corresponding to the Mcl-1 loop domain between residues 194 and 204 (TAT-T191-A204) or scramble control (TAT-Scr) peptide were synthesized by Biomatik USA (Wilmington, DE). For detection, peptides were labeled with FITC at the C terminus. HCT116 p53+/− cells were incubated with 5 or 10 μM TAT-T191-A204 or TAT-Scr for 60 min, followed by treatment with or without doxorubicin for 24 h.

**Immunoblotting**—Western blot analyses were performed as described previously (25). The membranes were visualized using ECL reagents (GE Healthcare) or the WesternBright Quantum kit (Advansta, Menlo Park, CA). The primary antibodies used for Western blotting were anti-Mcl-1 (catalog no. D35A5, rabbit, dilution of 1:1000, Cell Signaling Technology), anti-Mcl-1 (catalog no. Sc-966, Santa Cruz Biotechnology), and anti-Mcl-1 (catalog no. K-20, rabbit, dilution of 1:1000, Santa Cruz Biotechnology). Mouse anti-β-actin (Santa Cruz Biotechnology) at a dilution of 1:10,000 was used as a loading control.

**Immunofluorescence**—Immunofluorescence was performed as described previously (11). Anti-PML (catalog no. sc-966, mouse, 1:100 dilution) was purchased from Santa Cruz Biotechnology. Anti-γH2AX (Ser-139, mouse, 1:100) was purchased from BioLegend, and anti-Ki67 (catalog no. 550609, mouse, 1:100) was purchased from BD Biosciences. Cells were incubated with a goat anti-mouse (clone Poly4043) or a donkey anti-rabbit (clone Poly4064) secondary antibody conjugated with Cy3 (BioLegend, San Diego, CA) for 1 h in the dark, washed with PBS, and mounted on microscope slides using Vectashield mounting medium containing DAPI for fluorescence (Vector Laboratories, Burlingame, CA). Images were captured on a Leica SP2 confocal microscope using the appropriate filter sets.

**PML and γH2AX Focus Quantification**—Ten representative fields were selected randomly for the quantification of PML and γH2AX nuclear body formation. The number of foci present in
each cell nucleus was counted manually in 30 transfected and drug-treated cells as well as in 30 transfected but not drug-treated cells using a Leica DM5500 B fluorescence microscope at ×40 oil immersion.

**Senescence β-Galactosidase Assays**—48 h post-transfection, or after 6 days of treatment/no treatment with drugs, cells were assayed for senescence-associated (SA) β-gal expression as described previously (26). Briefly, cells were washed and fixed with 2% paraformaldehyde (Fisher Scientific) for 5 min at room temperature. Cells were then incubated in the dark for up to 16 h in a staining solution containing 1 mg/ml X-gal (Gold Biotechnology) in dimethylformamide (Acros Organics), 40 mM of a 0.2 M citric acid/sodium phosphate buffer (pH 6.0), 5 mM potassium ferrocyanide (Sigma), 5 mM potassium ferricyanide (Sigma), 150 mM sodium chloride, and 2 mM magnesium chloride. Stained cells were then visualized under an inverted bright-field microscope. Ten representative fields were selected randomly for the quantification of β-gal-positive cells as a percentage of the total cell number. For tissue analysis, fresh-frozen tissue samples were cut into 5 μm sections, fixed with 1% paraformaldehyde for 1 min, and washed with PBS, followed by overnight incubation with SA β-gal staining solution.

**Cell Proliferation Assays**—The proliferative capacity of cells was determined by a BrdU cell proliferation assay kit (EMD Millipore) or by ki67 immunohistochemical staining according to the instructions of the manufacturer. For the BrdU incorporation assay, cells were treated with or without BrdU-containing medium for 2 h. After the addition of goat anti-mouse IgG peroxidase-conjugated secondary antibody, substrate, and stop solution, the amount of BrdU was determined. Cultured cells without BrdU treatment were used as controls for nonspecific binding. The amount of BrdU incorporation in the proliferating cells was expressed as optical density mean values in the presence of BrdU—optical density mean values without BrdU ± S.D. Analyses were performed in triplicate.

**Apoptosis Assays**—Apoptosis assays were done using the BioLegend FITC Annexin V apoptosis detection kit with 7-aminoactinomycin D (7-AAD) according to the protocol supplied with the kit. HeLa cells transiently transfected with the indicated constructs were either left untreated or treated with 50 μM etoposide for 24 h and stained with Annexin-V (2.5 μg/ml) and 7-AAD (5 μg/ml) for analysis by flow cytometry on a FACSCalibur (BD Biosciences). Data were analyzed using the FlowJo data analysis software package.

**Tumor Xenograft Studies**—The HCT116 or HCT116 shMcl-1 cell lines were stably transfected with plasmids expressing vector, full-length Mcl-1, or Mcl-1 Δ1–157, Δ208–350, or Δ198–207. 1 × 10⁷ cells/mouse were implanted subcutaneously in the right dorsal flank of female athymic nude mice (National Cancer Institutes). Starting on day 10, a group of mice carrying vector, wild-type Mcl-1, or the indicated Mcl-1 constructs received 1.2 mg/kg doxorubicin via intraperitoneal injection every 3 days. For the spontaneous senescence assay, constructs were stably expressed in HCT116 shMcl1 cells and implanted subcutaneously into the flanks of athymic nude mice and left untreated. The length and width of the tumor were measured with calipers every third day, and the tumor volume was calculated as tumor volume = (length × width²) / 2. Tumor tissues from mice were fixed overnight in cold 4% paraformaldehyde (prepared in PBS) at 4 °C, followed by incubation in cold 30% sucrose/PBS solution for 24 h before embedding in optimal cutting temperature medium (Tissue-Tek) on dry ice and storage at −80 °C. Primary antibodies used for immunohistochemistry were anti-Mcl-1 (catalog no. Sc-966), anti-Mcl-1 (catalog no. K-20, rabbit, dilution of 1:1000), and anti-PML (catalog no. sc-966, mouse, 1:100 dilution), all of which were purchased from Santa Cruz Biotechnology. Anti-yH2AX (Ser-139, mouse, 1:100) was purchased from BioLegend, and anti-Ki67 (catalog no. 550609, mouse, 1:100) was from BD Biosciences. All animals were maintained in pathogen-free animal facilities at the Cleveland Clinic Lerner Research Institute, and all procedures were performed under Institutional Animal Care and Use Committee-approved Protocols 2010-0350 and 2013-1143.

**Human Tissue Specimens**—All human tissue specimens were collected under an Institutional Review Board-approved protocol at the Cleveland Clinic. Informed consent was obtained from all subjects. Fresh-frozen human CRC tumors (collected from different patients either before or after chemotherapy), liver metastases from two patients, or normal tissues obtained from a site distal to the primary colon tumor were sectioned at 5 μm thickness. Slides were fixed with 4% paraformaldehyde, air-dried, and stored at −20 °C until use. Immunohistochemical staining was carried out using the avidin-biotinylated peroxidase method with Vectastain ABC kits (Vector Laboratories). The primary antibodies anti-Mcl-1 (rabbit, 1:100), anti-PML (mouse, 1:100, Santa Cruz Biotechnology), anti-Ki67 (rabbit, 1:100, BD Biosciences), and anti-capase-3 (rabbit, 1:100, Cell Signaling Technology) were incubated overnight at 4 °C, and signals were visualized using 3,3′-diaminobenzidine tetrahydrochloride as described previously (9). Slides were counterstained with Mayer’s hematoxylin and viewed using a Nikon E400 microscope.

**Statistical Analysis**—Data are presented as mean ± S.D. or S.E. and are inclusive of at least two separate experiments. Differences between various experimental groups were calculated using Student’s t test, in which p < 0.05 was considered significant. Correlations between Mcl-1 mRNA expression in normal versus malignant CRC tissue and patient survival were determined using the Oncomine database.

**Results**

**Mcl-1 Inhibits CIS Using a Mitochondrion-independent Mechanism**—Most of the known prosurvival activities of Mcl-1 occur at the mitochondrial. Studies found that the N-terminal 79 amino acids and the C-terminal 23 amino acids of Mcl-1 are important for its mitochondrial localization (27). We generated mutants of Mcl-1 devoid of either domain, Δ1–79 or Δ328–350 (Fig. 1A). Analysis of the Δ1–79 and Δ328–350 mutants by Western blot showed them to be of the appropriate size (Fig. 1B). Similar to previous reports, both of these mutants had reduced mitochondrial localization with a diffuse cellular pattern in contrast to WT-Mcl-1, which is depicted in Fig. 1C and quantified in Fig. 1D (18, 27). Also, we consistently observed significant changes in mitochondrial morphology in Δ1–79 and
Δ328–350 mutant-expressing cells that closely resemble those reported previously using similar Mcl-1 mutants (27). Using multiple measures of senescence, we reveal that these mutants inhibit CIS similar to WT-Mcl-1 (Figs. 1, E–H). These data are in contrast to the important role these mitochondrially localizing domains play in the antiapoptotic function of Mcl-1 (27).

Posttranslational Modification Sites and BH Domains Are Dispensable for the ability of Mcl-1 to Inhibit CIS—Having identified the unique CIS-related functions of Mcl-1, we next studied whether any of the unique structural characteristics of Mcl-1, shown to be critical in apoptosis regulation, could account for its anti-CIS abilities. Within the unique N terminus...
of Mcl-1 are two major and two minor proline, glutamic acid, serine, and threonine (PEST) sequences that are targets of phosphorylation and contribute to the relatively short half-life of Mcl-1 (2–3 h) (28). We designed alanine substitutions to remove PEST phosphorylation sites from Mcl-1, including those that stabilize (e.g. Thr-92 and Ser-121), or destabilize
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(Ser-159, and Thr-163) the protein upon phosphorylation (17). These constructs were transiently transfected into HCT116 p53−/− cells with stable knockdown of Mcl-1. The site-directed mutagenesis is depicted in Fig. 2Ai, and expression of the constructs is shown in Fig. 2Aii. Note that Mcl-1 can appear on a Western blot as a singlet or doublet, as in Fig. 2Aii. This has been reported to be due to a slight truncation at the N terminus, which appears to be of little overall physiologic consequence (29). Fig. 2, Aiii–Avi, depicts multiple measurements of senescence, revealing that, although there are small but significant differences between the mutants and WT Mcl-1 in some assays (particularly in SA β-galactosidase activity), each mutant displayed dramatic antisenescence activity. We next tested large N-terminal and C-terminal deletions of Mcl-1 (Fig. 2, B and C, respectively). In Fig. 2B, we employed deletion mutants, Δ1–127 and Δ1–157, that provided constructs equivalent to post-caspase cleavage forms of the molecule that we and others showed abrogate the antiapoptotic function of Mcl-1 (30). We observed that the constructs migrated at the expected size in Western blots and had anti-CIS abilities similar to WT Mcl-1 (Fig. 2, Bi–Bvi). In Fig. 2C, we employed a mutant devoid of the known three Bcl-2 homology (BH) domains (Δ208–350) of Mcl-1 and, therefore, lacking its canonical binding cleft (the BH3 binding pocket). Similar to Fig. 2B, Fig. 2C demonstrates that a construct of Mcl-1 without the known functionally important C-terminal domains migrates appropriately on Western blot (Fig. 2Cii). Of note, the Western blot shown was repeated several times, and some of them do or do not show additional degradation products, as can be seen in this figure. Regardless, the functional assays were consistent, and the Δ208–350 mutant inhibits CIS similar to (PML, YH2AX) or better than (SA β-gal) WT Mcl-1 (Fig. 2, Ci–Cvi). In summation, these results suggest that much of the Mcl-1 protein is dispensable for its antisenescence activity and that the residues between 158 and 207 are necessary for this function.

Mcl-1 Contains 4 Residues within a Loop Domain That Are Critical for CIS Resistance—To determine which of the 50 residues not covered by previous mutations were associated with the anti-CIS activity of Mcl-1, we generated five separate 10-amino acid deletion mutants (158–167, 168–177, etc.; Fig. 3Aii). Fig. 3Aii illustrates expression of these mutants in HCT116 p53−/− cells expressing a stable shMcl-1. Fig. 3, Aiii–Avi, reveals, using multiple senescence assays, that residues 188–207 contain the domain responsible for the anti-CIS activities of Mcl-1. We then performed alanine substitutions on the majority of residues, 188–207, and expressed those in HCT116 p53−/− shMcl-1 cells. Fig. 3, Bi–Bvi, illustrates that alanine substitution at sites 194, 197, 198, and 203 caused near complete abrogation of the anti-CIS activities of Mcl-1. Moreover, all four residues are contained in a poorly described loop domain within Mcl-1, as shown in Fig. 3Bv (image on the basis of previously described structural studies (31)). Residues 194 and 197 are known to be ubiquitinated by the E3 ubiquitin ligase Mcl-1 ubiquitin ligase E3 (MULE), which requires an intact Mcl-1 N terminus (32) and limits the antiapoptotic functions of Mcl-1 (16). It is unlikely that MULE and, for that matter, ubiquitination are critical for senescence modulation because altering these sites would be expected to cause a gain of function, unlike what we observed. We also tested various Mcl-1 constructs that have altered senescence (Δ198–207, P198A) and apoptosis (Δ208–350) functions as well as the wild type for their ability to inhibit apoptosis. In Fig. 3, Ci–Cvi, we expressed these constructs in HeLa cells, revealing that only the antiapoptotic-deficient mutant had impaired apoptosis inhibition similar to a vector control. This indicates that alterations to the loop domain do not affect the major three-dimensional structures of Mcl-1 required for apoptosis inhibition. Finally, we identified three cell lines that are susceptible to senescence. Two of them, CCF2968 (melanoma, Fig. 4A) and HN572 (human papillomavirus-positive head and neck cancer, Fig. 4B), are low-passage cell lines from patients treated at our institution. The HN572 and SW480 (colon cancer, Fig. 4C) cell lines both possess impaired p53 function. In each case, the relative anti-CIS activity (or lack thereof) of each construct was consistent with previous results regardless of the type of cells in which they were expressed (Fig. 4, Ai–Ci).

The Loop Region of Mcl-1 Is Critical to Tumor CIS Resistance and Growth Potential in Vivo—In our previous work, we developed an in vivo xenograft model of CIS using HCT116 cells (Mcl-1-proficient) (9). We found that, similar to in vitro studies, CIS is blocked in cells with overexpression of WT Mcl-1 in vivo, which is recapitulated in Fig. 5Aii. To test whether the domain within Mcl-1 that is responsible for CIS resistance in vitro accounts for its activity in vivo, we stably expressed three deletion mutants of Mcl-1 in HCT116 cells, Δ1–157, Δ208–250, and Δ198–207, and implanted them into athymic nu/nu mice. Fig. 5, Ai–Av, illustrates that, similar to our in vitro data, neither the N-terminal 157 amino acidsnor the 142 C-terminal residues are required for the CIS resistance of Mcl-1 in vivo, although residues 198–207 containing the loop domain are

FIGURE 2. Posttranslational modification sites and BH domains are dispensable for the ability of Mcl-1 to inhibit CIS. Ai, schematic of the wild-type Mcl-1 protein. Arrows indicate alanine substitutions of important phosphorylation sites. P, post domain (proline, glutamic acid, serine, and threonine). MP, minor PEST domain. TM, transmembrane domain. Aii, Western blot of Mcl-1 protein levels after transient transfection of the indicated constructs in HCT116 p53−/− shMcl-1 cells. HCT116 p53−/− shMcl-1 cells were transiently transfected with vector control, WT Mcl-1, or the indicated constructs and then either treated with doxorubicin or left untreated. Aiii–Avi, data showing a decrease in β-gal (Aiii), PML nuclear body (Avi), and YH2AX nuclear body formation (Avi) and an increase in ki67 staining (Avi) in cells expressing the phosphomimetic mutants of Mcl-1. Dax, doxorubicin. Bi, schematic of N-terminal deletions of Mcl-1 equivalent to post-caspase cleavage. HCT116 p53−/− shMcl-1 cells were transiently transfected with vector control, WT Mcl-1, or Δ1–127 or Δ1–157 constructs. Bii, Western blot of Mcl-1 protein levels after transfection of the indicated constructs. Biii–Bvi, quantitative analysis of CIS in N-terminal deletion mutants as assessed by β-gal activity (Biii), staining with antibodies for PML (Biv) and YH2AX (Bv) nuclear bodies, and BrdU staining (Bvi). Ci and Cii, schematic of the Δ208–350 construct. HCT116p53−/− shMcl-1 cells were transiently transfected with empty pcDNA3.1/myc-His vector, WT-Mcl-1 (non-tagged), or pcDNA3.1/myc-HisΔ208–350 constructs and verified by Western blot (Ciii). Ciii–Cvi, quantitative analysis of CIS as assessed by β-gal staining (Ciii), PML nuclear body formation (Civ), YH2AX nuclear body formation (Cvi), and BrdU staining (Cvi). All data are representative of three independent experiments. NS, no statistical differences between the indicated constructs and WT Mcl-1 in doxorubicin-treated cells. *p < 0.05 for doxorubicin-treated cells, comparing those expressing indicated transfected constructs with WT Mcl-1. Error bars represent mean ± S.D. Quantitative data are inclusive of at least three independent experiments.
quite critical for this function. We have also shown previously that merely stably knocking down Mcl-1 expression in untreated HCT116 cells (regardless of p53 status) caused tumor growth inhibition in vivo, with the tumor cells demonstrating many features of senescence but not apoptosis. To assess whether the ability of Mcl-1 to inhibit this form of spontaneous senescence is dependent on the same domain that is responsible for its anti-CIS activities, we stably transfected WT, Δ1–157, Δ208–250, or Δ198–207 constructs of the protein into Mcl-1-deficient HCT116 cells, which were then used to establish xenograft tumors. Fig. 5B reveals that it is the loop domain residues (198–207) that are responsible for the anti-senescence functions of Mcl-1. Fig. 5C shows the overexpression of the several Mcl-1 variants (versus vector control) whose growth curves are shown Fig. 5, A and B. We confirmed that the Mcl-1 staining was due to overexpression and not up-regulation of endogenous Mcl-1 by testing the Δ208–250 Mcl-1 variant with multiple antibodies, including one that cannot detect this construct (K-20) because of epitope loss (Fig. 5D). In addition, we assessed senescence levels by analyzing SAβgal activity using immunohistochemistry and staining of PML body and Ki67 expression and apoptosis induction by caspase-3 activity. Fig. 5C illustrates the results of these senescence and apoptosis assays in the CIS experiment (growth curves are shown in Fig. 5, Ai–Av). During CIS in vivo in cells expressing WT, Δ1–157, or Δ208–250 that had robust tumor growth, there is neither

FIGURE 3. Mcl-1 contains four residues in a loop domain that are critical for CIS resistance. Ai, schematic of five sequential 10-amino acid deletion mutants. Aii, Western blot of Mcl-1 protein levels after transfection of the indicated constructs. Aiii–Avi, quantitative analysis of CIS showing the sensitivity of the Δ188–197 and Δ198–207 mutants to doxorubicin treatment, as assessed by β-gal activity (Aiii), PML nuclear body (Aiv) and γH2AX nuclear body (Av) formation, and a decrease in Ki67 staining (Avi). B, HCT116p53−/− shMcl-1 cells transiently transfected with vector control, WT Mcl-1, or several single alanine substitutions. Bi, Western blot of Mcl-1 protein expression of the indicated constructs. Bii–Biv, quantitative analysis of CIS of alanine substitution mutants, K194A, K197A, P198A, and G203A as measured by increased β-gal activity (Bii), PML nuclear body formation (Biii), and decreased Ki67 staining (Biv). *, p < 0.05 for the indicated doxorubicin-treated constructs compared with doxorubicin-treated Mcl-1. Data are representative of three independent experiments. Error bars represent mean ± S.D. Bi, crystal structure of a portion of Mcl-1 with relative locations of residues Lys-194, Lys-197, Pro-198, and Gly-203 highlighted in relation to the C-terminal antiapoptotic binding groove bound to a proapoptotic BH3-only Bcl-2 family (yellow). Ci and Cii, HeLa cells were transiently transfected with the vector control, WT Mcl-1, or the Mcl-1 mutants Δ208–350, P198A, and R201A and were either left untreated or treated with 50 μM of etoposide and stained with Annexin-V and 7-AAD. Ci, Western blot of Mcl-1 protein levels after transfection of the indicated constructs. Cii, FACS analysis of Annexin-V and 7-AAD. *, p < 0.05 for the indicated etoposide-treated constructs compared with the vector control. Error bars represent mean ± S.D. Data are inclusive of three independent experiments.

FIGURE 4. Expression of Mcl-1 loop domain mutants in other CIS-sensitive cell lines. A–C, the indicated constructs were transiently expressed in CCF2968 (Ai), HNS72 (Bi), and SW480 (Ci) cell lines. Ai, Bi, and Ci, construct expression was verified by Western blot analysis. Ai–Avi, cells were left untreated or treated with doxorubicin (Dox), and CIS sensitivity was examined by PML (Aii, Bii, and Cii) and γH2AX nuclear body formation (Aiii, Biii, and Ciii). Data are inclusive of three independent experiments. *, p < 0.05 for the indicated doxorubicin-treated constructs compared with doxorubicin-treated Mcl-1. Error bars represent mean ± S.D.
significant senescence nor apoptosis. In contrast, cells expressing the \( \Delta 198-207 \) variant under CIS conditions not only displayed delayed growth (Fig. 5Ai) but also contained a significant expression of senescence markers (high SA-\( \beta \)gal activity, a large number of PML bodies, and low Ki67 expression) without measurable apoptosis activity (Fig. 5Aiv). Although not shown, the spontaneous senescence groups demonstrated nearly identical results. The sum of these results indicates that the loop domain of Mcl-1 is not just responsible for preventing CIS in tumors but drives these cancer cells to grow under \textit{in vivo} conditions.

The Loop Domain in Mcl-1 Can Be Functionally Blocked through Dominant Negative Inhibition—We next tested the possibility that functionally altered constructs of Mcl-1 might compete with endogenous Mcl-1. We expressed variants that can (R201A) or cannot (P198A, G203A) resist CIS in HCT116 \textit{p53} \(-/-\) cells with endogenous Mcl-1 expression. Surprisingly, cells expressing endogenous Mcl-1 transfected with specific loop-impaired variants showed significant sensitivity to CIS (Figs. 6, B–D). This indicates that these mutants can act in a dominant negative fashion on the endogenous protein. Additionally, because others have successfully designed cell-permea-
able peptide inhibitors on the basis of the specific functional domain of their target protein and even showed that some can act as dominant negative inhibitors (33), we synthesized a peptide on the basis of the 14 residues of the loop domain of Mcl-1 and combined it with an N-terminal HIV-TAT sequence for cell permeability and a C-terminal FITC for identification (TAT-Mcl-1, Fig. 6E). In comparison with a similar peptide with the loop domain residues scrambled (TAT-Scr), there was

![Figure 6](image-url)

**FIGURE 6.** The Mcl-1 loop domain can be functionally blocked through dominant negative inhibition. A, Western blot for construct expression in endogenous Mcl-1-expressing HCT116 p53−/− cells transiently transfected with the vector control, WT Mcl-1, or various Mcl-1 constructs. Data are representative of two independent experiments. B–D, quantitative analysis of CIS in cells transiently transfected with the vector control, WT Mcl-1, or various Mcl-1 constructs and analyzed for β-gal activity (B), PML nuclear body formation (C), or γH2AX nuclear body formation (D). *, p < 0.05 for the indicated doxorubicin-treated constructs compared with doxorubicin-treated Mcl-1 cells. Error bars represent ± S.D. E, schematic of cell-permeable peptides synthesized to contain an N-terminal HIV-TAT sequence conjugated to a peptide sequence corresponding to the Mcl-1 loop domain between residues 194 and 204 (TAT-Mcl-1) or scramble control (TAT-Scr) and a C-terminal FITC group. F and G, quantitative analysis of CIS of cells preincubated with TAT-Mcl-1 or scramble control at the indicated concentrations and left untreated or treated with doxorubicin via PML (F) and γH2AX nuclear body formation (G) formation. *, p < 0.05 for TAT-Mcl-1 compared with TAT-Scr at the indicated concentrations. NS, no statistical differences between the indicated constructs and WT Mcl-1 in doxorubicin-treated cells. Error bars represent mean ± S.D. Data are inclusive of three independent experiments.
A Unique Domain of Mcl-1 Regulates Senescence Inhibition

A dose-dependent increase in sensitivity to CIS in HCT116 p53−/−, Mcl-1-proficient cells treated with TAT-Mcl-1 (Fig. 6, F–H).

Mcl-1 Is Associated with Colon Cancer and Reduced Levels of Senescence in Patients—Having worked extensively with the HCT116 CRC cell line, we wanted to correlate our experimental findings with results obtained from patients with this type of cancer. Fig. 7Ai shows that multiple CRC cell lines express high levels of Mcl-1 compared with primary colonic epithelium (CRL-1459) cells. Fig. 7, Aii and Aiii, illustrate both at the protein (representative of multiple immunohistochemistry samples) and mRNA levels (using Oncomine database software with the Hong dataset (34) (Fig. 7Aii)) that Mcl-1 expression is significantly higher in CRC tissue than in normal colon epithelium. CRC is treated distinctly when it is of rectal (chemotherapy before resection) or colon (resection before chemotherapy) origin. As a result, our colorectal cancer tissue bank contains both pre- and post-chemotherapy specimens from different...
patients. Fig. 7, Bi–Bii, shows that, in CRC tissues collected from separate patients either before or after chemotherapy, the level of Mcl-1 expression throughout the tissue is often heterogeneous (as measured by immunohistochemical staining) and features regions of high and low expression. Significantly, we consistently found that areas of high Mcl-1 expression were associated with low to undetectable levels of senescence markers and vice versa (using SA βgal and PML body formation as readouts). In all cases, there was little detected apoptosis (as measured by caspase-3 activity). Fig. 7Biii is a representative of two colon cancer liver metastases (an aggressive variant) from separate patients, showing the commonly observed homogeneous high expression of Mcl-1 accompanied by low expression of senescence markers. Fig. 7Biv was generated from the Oncomine database software using the Staub dataset (35) and is consistent with literature associating Mcl-1 expression with a poor prognosis in many types of cancer, including colon cancer (36). These data are also consistent with studies showing a better prognosis associated with high levels of senescence observed in multiple forms of cancer before and after treatment.(20). To date, our work represents the first association of Mcl-1 levels and senescence in human cancer.

Discussion

Encouraging results using small molecule inhibitors in both murine studies and human clinical trials have identified antiapoptotic Bcl-2 family members, especially Mcl-1, as attractive targets for cancer therapy (37). More recent successes with Bcl-2 inhibitors and other clinically effective small molecules now indicate that, in addition to promoting tumor cell apoptosis, some of these interventions also inhibited tumor growth by mediating tumor cell senescence (38, 39). We too have reported recently that targeting Mcl-1 inhibits tumor cell progression by affecting both its antiapoptotic and antisenescence activities (11). These findings are extremely relevant given that tumor cell senescence is increasingly being observed in patients who were treated successfully for a variety of cancers (20, 40, 41). This suggests an important opportunity to design drugs capable of inducing CIS in cancer by targeting molecules like Mcl-1.

The mitochondrion is the organelle most commonly associated with the apoptosis-related activity of Bcl-2 family members, including Mcl-1 (27). We generated mutants of both the N and C termini that have been shown previously to reduce the mitochondrial localization and function of Mcl-1 (18, 27). Similar to these studies, our Δ1–79 and Δ328–350 mutants also have reduced mitochondrial localization, although their ability to confer resistance to CIS remains unaffected. Whether this observation reflects the ability of Mcl-1 to resist CIS outside of the mitochondria or that the reduced amount of mitochondrial Mcl-1 remains sufficient for anti-CIS activity remains to be determined. Through further mutagenesis, we show that the additional N-terminal post-translational regulation sites of Mcl-1 (phosphorylation and caspase cleavage) have little role in senescence modulation. We have shown previously that, under CIS conditions, Mcl-1 levels remain stable (11). Because many of these regulatory sites under apoptotic conditions regulate the half-life and, therefore, antiapoptotic activity of Mcl-1, it is possible that, under alternative conditions, e.g. CIS, the signals for Mcl-1 posttranslational modification are distinct. If, under senescence conditions, these signals are different from those that are apoptotic, then this would explain why loss of these regulatory domains may not have a major effect on CIS inhibition. More surprising was our observation that all three known C-terminal BH domains were dispensable for the anti-CIS activity of Mcl-1 in vitro and in vivo. Untreated xenograft tumors with knockdown of endogenous Mcl-1 but transfected to express Mcl-1 lacking the BH domains grew in mice at elevated rates in comparison with tumors transfected with vector control, which grew slowly and were observed to have high levels of spontaneous senescence (not apoptosis). These BH domains make up the canonical antiapoptotic binding pocket/ groove of Mcl-1, which is thought to be its main antiapoptotic and, therefore, protumor, aspect (10, 12, 14, 42). Our data imply that an additional domain within Mcl-1 must be responsible for its antisenescent function, which could yield a new set of important interacting proteins.

Indeed, through extensive mutagenesis, we were able to identify that an internal loop domain of Mcl-1 is responsible for the anti-CIS functions of the protein both in vitro and in vivo. Moreover, xenograft tumors with Mcl-1 knocked down and transfected with Mcl-1 mutants devoid of a large portion of this loop domain were similar to the vector control, with slow growth associated with a form of spontaneous senescence but not apoptosis. Interestingly, loop domains of Bcl-2 and Bcl-XL, which are much larger than those of Mcl-1, contain many important phosphorylation sites critical in the regulation of those proteins (43–45). In contrast, little is known about the loop domain of Mcl-1, which, on the basis of our data, contains no essential residues associated with function-altering phosphorylation sites. We now know that residues 194, 197, 198, and 203 are critical. It is noteworthy that residue 198 within this Mcl-1 loop domain is a proline that is evolutionally conserved at least as far back as zebrafish.

We were also able to develop a proof of concept for the probable efficacy of Mcl-1 inhibition using a cell-permeable peptide targeting the loop domain. The peptide we designed converts CIS-resistant Mcl-1 wild-type tumors into tumors demonstrating CIS sensitivity. Although this form of therapy may have direct clinical applicability, it can also be used to design other effective small-molecule inhibitors, a strategy already having clinical success in some cancers (46).

The HCT116 CRC cell line was the first cell line to have somatic deletions in senescence-related proteins and, therefore, is used widely as a model for CIS (22, 47, 48). We therefore decided to translate our findings and employed tissues collected from human CRC patients, showing, for the first time, that local levels of Mcl-1 expression were associated inversely with levels of senescence. Whether these two prognostic indicators (Mcl-1 and senescence) are linked from a treatment efficacy standpoint has yet to be determined. However, they may represent new biomarkers to optimize future cancer therapies.

In summary, our data reveal unique features of Mcl-1 that can be targeted to improve cancer therapy. Our findings indi-
cate that Mcl-1 can inhibit both apoptosis and senescence through distinct molecular domains, likely explaining the limited clinical successes of drugs targeting only its antiapoptotic functions. By accounting for these newly discovered structural characteristics of Mcl-1, ideal therapies can be designed to target one of the most important pro-oncogenic agents in all of human cancer.

Author Contributions—A. D. performed all of the experiments with support from L. W. P., who prepared the cell lines for xenograft tumors, injected them into nude mice, measured tumor volumes, and performed apoptosis assays. C. S. T. generated the alanine substitution mutants. X. L. provided substantial guidance for the design and evaluation of TAT-peptide constructs and proofread the manuscript. M. F. K. and J. D. provided human CRC specimens. A. D. and B. R. G. wrote the manuscript, and C. S. T. and L. W. P. edited and revised the paper. All authors reviewed and approved the final version of the manuscript.

Acknowledgments—We thank Simon Schlanger for help with creating the 3D images of Mcl-1, and Dr. William Flavahan for assistance with the Oncomine database analyses.

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