Inhibition of Wild-Type p53-Expressing AML by the Novel Small Molecule HDM2 Inhibitor CGM097

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Abstract

The tumor suppressor p53 is a key regulator of apoptosis and functions upstream in the apoptotic cascade by both indirectly and directly regulating Bcl-2 family proteins. In cells expressing wild-type (WT) p53, the HDM2 protein binds to p53 and blocks its activity. Inhibition of HDM2:p53 interaction activates p53 and causes apoptosis or cell-cycle arrest. Here, we investigated the ability of the novel HDM2 inhibitor CGM097 to potently and selectively kill WT p53-expressing AML cells. The antileukemic effects of CGM097 were studied using cell-based proliferation assays (human AML cell lines, primary AML patient cells, and normal bone marrow samples), apoptosis, and cell-cycle assays, ELISA, immunoblotting, and an AML patient–derived in vivo mouse model. CGM097 potently and selectively inhibited the proliferation of human AML cell lines and the majority of primary AML cells expressing WT p53, but not mutant p53, in a target-specific manner. Several patient samples that harbored mutant p53 were comparatively unresponsive to CGM097. Synergy was observed when CGM097 was combined with FLT3 inhibition against oncogenic FLT3-expressing cells cultured both in the absence as well as the presence of cytoprotective stromal-secreted cytokines, as well as when combined with MEK inhibition in cells with activated MAPK signaling. Finally, CGM097 was effective in reducing leukemia burden in vivo. These data suggest that CGM097 is a promising treatment for AML characterized as harboring WT p53 as a single agent, as well as in combination with other therapies targeting oncogene-activated pathways that drive AML.

Introduction

The p53 proto-oncogene is a transcription factor that controls the expression of a multitude of target genes involved in DNA repair, apoptosis, and cell-cycle arrest, which are all important phenomena counteracting the malignant growth of tumors. Dubbed “guardian of the genome,” p53 is thus critical for maintaining genetic stability, prohibiting defective cells from dividing, and preventing tumor development (1).

While higher p53 expression correlates with induction of apoptosis and lower p53 expression correlates with inhibition of cell-cycle progression (2–3), it is the cooperative binding of p53 to apoptosis-inducing genes versus cell-cycle arrest genes that substantially contributes to cellular fate (4). p53-induced apoptosis is mediated by proapoptotic factors, including phosphol-12-myristate-13-acetate-induced protein 1 (PMAIP1; NOXA), and p53-upregulated modulator of apoptosis (PUMA), which bind to antiapoptotic proteins, including Bcl-2 family members (5–6).

Mutations in p53 that disrupt its activity are found in more than 50% of all human malignancies, which makes p53 the most frequently mutated gene in transformed cells in humans. In cancers in which the p53 gene is not mutated, the function of the p53 pathway is often suppressed through mechanisms that affect its stability and activity. One such mechanism is overexpression or deregulation of MDM2. MDM2, for which the human ortholog is known as HDM2, is an E3 ubiquitin ligase which, by direct binding (8), negatively regulates p53 through its ubiquitination and subsequent proteasome degradation (7, 8).

Different strategies to restore p53 function in tumors, including acute leukemia, have been attempted. These include the design of antagonists (small molecule and peptides) to prevent interaction of WT p53 with its negative regulators, such as...
HDM2 (9–13), design of molecules that directly reactivate mutant p53 (14), and exogenous WT p53 expression, for example, via adenovirus-mediated gene transfer (reviewed in ref. 15). Two studies in transgenic mice, in which p53 expression was reversibly switched on and off, have independently shown that restoration of p53 function can lead to tumor regression in vivo, indicating that reactivating p53 is a promising therapeutic strategy (16–17).

Inhibition of the p53–HDM2 protein–protein interaction using small molecules as a means to antagonize negative regulators of p53 and thus activate p53 has been focused on targeting the p53-binding pocket of HDM2. The first HDM2 inhibitor to be developed was 4,5-dihydroimidazoline, also known as Nutlin (9). Further structure-based optimization of Nutlin compounds yielded RG7112 and RG7388 (Roche). These compounds have recently been shown to lead to pharmacologic p53 pathway activation and downstream signaling in human tumor tissue (18–19) and demonstrated initial signs of efficacy in patients with solid and hematologic malignancies (20). Several other chemical classes of HDM2 inhibitors have been developed, among which those that advanced into phase I clinical development include AMG232 (Amgen), MI-773 (Sanoﬁ), DS-30326 (Daiichi Sankyo), and MK842 (Merck; refs. 13, 21).

Early clinical trials with advanced-stage HDM2 inhibitors was limited by toxicity of these compounds (reviewed in ref. 13). Even though HDM2 has been validated as a promising target for drug development, previous results warrants the identiﬁcation and development of novel, more potent and efficacious HDM2 inhibitors with less toxic side effects. Here, we introduce the Novartis HDM2 inhibitor, CGM097, as a potent inhibitor of WT p53-expressing AML that has the potential to be used alone or in combination with conventional therapies, such as Ara-C (cytarabine), or other oncogene-targeted therapies for AML.

Materials and Methods

Cell lines and cell culture

OCI-AML3, OCI-AML5, P31-FUJ, SKM-1, NOMO-1, and NB4 were obtained from Dr. Gary Gilliland (Merck Research Laboratories, Boston, MA). HEL, HL60, KG-1, Kasumi-1, KU812F, IP397, MONOMAC6, and SKNO-1 were purchased from the ATCC. MOLM-13 (DSMZ; German Resource Centre for Biological Material, was engineered to express luciferase fused to neomycin phosphotransferase (pMMP-LucNeo) by transduction with a VSVG-pseudotyped retrovirus as previously described (22). MOLM-14 (23) was provided by Dr. Scott Armstrong, Dana Farber Cancer Institute (Boston, MA).

OCI-AML3 cells were cultured in α-MEM media (Mediatech, Inc) + 10% FBS + 2% L-glutamine + 1% penicillin/streptomycin. OCI-AML5 cells were cultured in RPMI + 10% FBS + 2% L-glutamine + 1% penicillin/streptomycin, and supplemented with GM-CSF. All other lines were cultured in RPMI (Mediatech, Inc.) + 10% FBS + 2% L-glutamine + 1% penicillin/streptomycin. Cells were cultured in 5% CO2 at 37°C at a concentration of 2 × 105 to 5 × 106.

We have authenticated the following cell lines through cell line short tandem repeat (STR) proﬁling (DDC Medical): MOLM14, NOMO-1, HEL, SKM-1, OCI-AML3, and NB4. All cell lines matched >80% with lines listed in the DSmZ Cell Line Bank STR Proﬁle Information. Other cell lines expressing mutated RAS or wild-type RAS were sequenced or tested with pharmacologic inhibitors for validation of cell line integrity (24). All cell lines were obtained between 2000 and 2015.

Chemical compounds and biologic reagents

CGM097 and PKC412 were synthesized by Novartis Pharma AG. AZD6244 and AC220 were purchased from MedChem Express Co. Ltd. Compounds were initially dissolved in DMSO to make 10 mmol/L stock solutions, and then were serially diluted to obtain ﬁnal concentrations for in vitro experiments.

AML patient cells

Frozen vials of bone marrow from AML patients identiﬁed as harboring mutant FLT3 were thawed prior to processing. Mononuclear cells were isolated from normal bone marrow by density gradient centrifugation through Ficoll-Plaque Plus (Amersham Pharmacia Biotech AB) at 2,000 rpm for 30 minutes, followed by two washes in 1 × PBS. Mononuclear cells were then tested in liquid culture (Iscove MDM, supplemented with 20% FCS). All bone marrow samples from AML patients were obtained under approval of the Dana-Farber Cancer Institute Institutional Review Board. Patient information is provided in Supplementary Table SI.

ELISA assay

MIC-1 ELISAs were performed using the Quantikine ELISA assay Human GDF-15 (R&D Systems) as follows: After culturing cells with DMSO or CGM097 for 2 to 3 days, supernatants were harvested and incubated in the assay plate for 2 hours. Bound protein was probed with a primary antibody against MIC-1, and absorption was measured on a spectrophotometer (Molecular Devices) following addition of secondary antibody conjugated to HRP.

Proliferation studies, apoptosis assays, and cell-cycle analysis

Cell counts for proliferation studies were obtained using the Trypan blue exclusion assay, as previously described (24). Error bars represent the SEM for each data point. Programmed cell death of inhibitor-treated cells was determined using the Annexin-V-Fluos Staining Kit (Boehringer Mannheim), as previously described (24). Cell-cycle analysis was performed as previously described (24).

Drug combination studies

For drug combination studies, compounds were added simultaneously at ﬁxed ratios to cells, and cell viability was determined by Trypan blue exclusion and expressed as the function of growth affected (FA) drug-treated versus control cells. Synergy was assessed by Calculys software (Biosoft), using the Chou–Talalay method (25). The combination index = [D]1/[D]1 + [D]2/[D]2, where [D]1 and [D]2 are the concentrations required by each drug in combination to achieve the same effect as concentrations [D]1 and [D]2 of each drug alone.

Colonies assays

Soft agar colony assays utilized an upper layer of 3% Noble agar (Difco) in IMDM (HyClone), supplemented with FCS and L-glutamine, and a lower layer of 5% Noble agar in IMDM medium, also supplemented with FCS and L-glutamine, in each well of a 24-well plate. Agar was liqueﬁed by microwaveing, and then heated at 55°C prior to cooling to 45°C before plating and solidifying. Colonies were allowed to grow for >2 weeks prior to counting.
Genotyping

Ten exons were amplified by PCR with exon-specific primers. PCR reactions were set up in 25 μL volume reaction containing 10 ng of genomic DNA, 0.4 μM of each primer and 1 U Taq DNA Platinum High Fidelity polymerase (Invitrogen cat#11304-029). After a denaturation step of 2 minutes at 94°C, the PCR consisted of 35 cycles of 30 seconds at 94°C, 30 seconds at 54°C, and 1 minute 30 seconds at 68°C. The resulting PCR products were evaluated on a 1% agarose gel. After purification of PCR products using an extraction kit (Qiagen), sequencing analysis in both directions was performed by either (i) using a BigDye Terminator v3.1 Cycle Sequencing Kit and 3730 DNA Analyzer (Applied Biosystems) with 10 pmol of sequencing primer (Boston University, Boston, MA), or (ii) in-house Sanger sequencing with 10 pmol of sequencing primer (Novartis Pharmaceuticals). Amplification and sequencing primers are shown in Supplementary Fig. S1.

Yeast-based p53 functional assay

The yeast-based p53 functional assay utilizes human p53 expressed in Saccharomyces cerevisiae, which activates ADE2 gene transcription (26). The assay tests the critical biologic function of p53 and can distinguish inactivating mutations from functionally silent mutations. Dr. Richard Iggo (Bergonie Cancer Institute, University of Bordeaux, France) kindly provided the yeast strain, YIG397, and pRDI-22 plasmid required for this assay. Briefly, p53 mRNA was isolated from cell lines or primary AML samples and reverse transcribed and amplified by PCR using the following primers:

- P3: 5'-ATT-TGA-TGC-TGG-CCC-CCG-AGG-ATA-TGAAG(S)C-3', 5'-ACC-CTT-TTT-GGA-CTT-CAG-GTG-GCT-GGA-GTG(S)G.

To test p53 status, yeast IG397 were cotransformed with unpurified RT-PCR product, linearized vector (pRDI-22 cut by HindIII and StudI), and carrier DNA by the lithium acetate unpuriication step of 2 minutes at 94°C, the PCR consisted of 35 cycles of 30 seconds at 94°C, 30 seconds at 54°C, and 1 minute 30 seconds at 68°C. The resulting PCR products were evaluated on a 1% agarose gel. After purification of PCR products using an extraction kit (Qiagen), sequencing analysis in both directions was performed by either (i) using a BigDye Terminator v3.1 Cycle Sequencing Kit and 3730 DNA Analyzer (Applied Biosystems) with 10 pmol of sequencing primer (Boston University, Boston, MA), or (ii) in-house Sanger sequencing with 10 pmol of sequencing primer (Novartis Pharmaceuticals). Amplification and sequencing primers are shown in Supplementary Fig. S1.

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To test p53 status, yeast IG397 were cotransformed with unpurified RT-PCR product, linearized vector (pRDI-22 cut by HindIII and StudI), and carrier DNA by the lithium acetate procedure (27), plated on synthetic minimal medium minus leucine plus adenine (5 μg/mL), and incubated for 2 to 3 days at 35°C. Specifically, gap repair of the plasmid with the PCR product leads to constitutive expression of the human p53 protein. Yeast-expressing repaired plasmids are selected on medium that lacks leucine but that contains just enough adenine for Ade- cells to grow. These yeast colonies, which contain mutant p53, are red. In contrast, colonies containing WT p53 are white. Red colonies are clearly identifiable after 2 to 3 days at 35°C; however, the color is more intense after an additional 2 days at 4°C.

In vivo study

Model development and efficacy study. The AML patient-derived mouse model was developed by intravenously implanting patient peripheral blood mononuclear cells (PBMC) into 8-week-old female NSG mice (The Jackson Laboratory; 5 million cells/mouse in 0.2 mL PBS). The model was serially transplanted twice in mice to acclimate in the Novartis NIBRI animal facility with access to food and water ad libitum for a minimum of 3 days prior to manipulation. Handling of mice and procedures described in this manuscript were in accordance with Novartis ACIC regulations and guidelines.

FACS analysis. Mouse blood (via tail vein nick) was collected into EDTA-coated capillary tubes (purple tops; Sarstedt P/N: 16.444.100) and 20 μL was aliquoted into a 96-well plate (BD P/N: 351177) containing 200 μL 1× RBC Lysis Buffer at room temperature for 5 minutes. The plate underwent centrifugation at 1,200 rpm for 5 minutes and supernatant was discarded. This step was repeated twice, after which cells were resuspended in 2% FBS in PBS, centrifuged at 1,200 rpm for 5 minutes and blocked with mouse and human Fc Blocking Reagents (1:20 dilution; Miltenyi P/N’s 130-092-575 and 130-059-091) for 10 to 30 minutes on ice. Samples were next incubated with either CD45-APC (eBioscience P/N: 17-9449-42) or APC isotype control (eBioscience P/N: 17-4714-42) antibody diluted in 2% FBS in PBS (1:200) for 30 to 60 minutes on ice in a dark room. Following antibody incubation, samples were washed twice in 2% FBS in PBS, resuspended in a final volume of 100 μL 2% FBS in PBS and processed for FACS analysis (BD FACSCanto II with 3 lasers—488-561-635 nm). Data were analyzed via Flowjo and reported as a percentage of hCD45+ cells within mouse PBMCs.

Tumor volume data analysis. Tumor burden was determined by hCD45+ cells present in mouse blood using FACS analysis methods described above.

Percent treatment/control (T/C) values were calculated using the following formula:

\[
\%T/C = 100 \times \frac{T - C}{D - C}
\]

where T is the mean tumor volume of the drug-treated group on the final day of the study; ΔT is the mean tumor volume of the drug-treated group on the final day of the study – mean tumor volume of the drug-treated group on initial day of dosing; C is the mean tumor volume of the control group on the final day of the study; and ΔC is the mean tumor volume of the control group on the initial day of dosing.

All data were expressed as mean and SEM. Significance was determined using a two-tailed unpaired Student t test. Statistical analysis was carried out using GraphPad Prism (GraphPad Software Inc.).

Results

CGM097 inhibits proliferation of WT p53-expressing AML via induction of apoptosis and inhibition of cell-cycle progression

We investigated the activity of the small-molecule HDM2 inhibitor, CGM097 (the chemical structure is depicted in

Published Online First July 23, 2015; DOI: 10.1158/1535-7163.MCT-15-0429
Fig. 1). against a panel of WT p53-expressing human AML cell lines (MOLM13, MOLM14, OCI-AML3, OCI-AML5) and mutant p53-expressing human AML cell lines (HL60, HEL, KG1, U937, NB4, MONOMAC6, Kasumi-1, SKNO-1, and P31; Fig. 1B; Supplementary Table SII). We found a positive correlation between WT p53 and good response to CGM097, whereas the opposite was true for mutant p53-expressing cells. CGM097 inhibition of proliferation of WT p53-expressing cell lines correlated with CGM097 induction of apoptosis (Fig. 1C and Supplementary Fig. S2) and G1 arrest (Fig. 1D and Supplementary Fig. S3), suggesting that programmed cell death and inhibition of cell-cycle progression contribute to the growth inhibitory effects of CGM097.

To investigate the potential of CGM097 to be used clinically, we tested the drug against 19 AML samples, the majority of which were characterized as expressing WT p53. We observed CGM097 to display substantial activity against the WT p53-expressing samples, with little-to-no activity against mutant p53-expressing samples (Fig. 2A; Supplementary Table SIII). The difference in response to CGM097 between WT p53-expressing samples and mutant p53-expressing samples was statistically significant ($P = 0.01$). In addition, 10 of 16 WT p53-expressing AML patient samples showed significantly higher responsiveness to CGM097 (near or over 50% drug-induced killing at 500 nmol/L) when compared with normal bone marrow samples, 4 out of 4 of which showed less than 50% killing (Fig. 2A and B; $P = 0.0004$). These data suggest that CGM097 is efficacious toward WT p53-expressing AML.

We adapted and utilized a simple yeast-based p53 functional assay (26) to validate the WT versus mutant p53 status in two WT p53-expressing AML patient samples that were tested for responsiveness to CGM097, as well as control cell lines expressing WT or mutant p53. As shown in Fig. 2C, this assay did, indeed, confirm the expression of WT p53 in these patient samples, a finding that also substantiates the utility of this assay as a complementary approach to genotyping.

CGM097 displayed a range of potencies against the WT p53-expressing AML patient samples. For example, all of the patient samples characterized as expressing WT p53 responded to 1,000 nmol/L CGM097 with significantly higher cell killing than mutant p53-expressing samples or KU812F cells (KU812F was used as a negative control for comparison due to expression of mutant p53 in this line). Among the WT p53-expressing patient samples, a large fraction were very sensitive, with IC50 values below 250 nmol/L, while others showed IC50 values of up to 1,000 nmol/L. This would indicate that even though CGM097 is highly active in some
WT 53 patients, the efficacy of this drug can be potentially influenced by other oncogenic drivers, signaling pathways or drug resistance mechanisms.

CGM097 selectively augments p53 activity in WT p53-expressing AML

The TGFβ superfamily cytokine, macrophage inhibitory cytokine-1 (MIC-1), serves as a secreted biomarker for p53 activation in cancer (28). An ELISA assay was employed to measure secreted MIC-1 in media collected from normal bone marrow cells or the WT p53-expressing AML cell line, MOLM14, treated with 0, 250, and 500 nmol/L CGM097. We observed CGM097 to selectively increase MIC secretion by MOLM14 cells but not normal human bone marrow, despite the expression of WT p53 in the normal bone marrow samples (Fig. 3A). Similarly, CGM097 selectively increased MIC-1 secretion by WT p53-expressing OCI-AML3 cells but not mutant p53-expressing HEL cells (Fig. 3B). These results further validate the target specificity of CGM097 towards WT, but not mutant, p53 in leukemic cells.

CGM097 synergizes with TKIs against oncogene-driven AML

We were also interested in testing the ability of CGM097 to synergize with a FLT3 inhibitor, presuming dual suppression of oncogenic FLT3 and induction of tumor suppressive WT p53. To address this, we first used FLT3-ITD–expressing MOLM13 cells, the proliferation and soft-agar colony growth of which are potently inhibited by CGM097 (Supplementary Fig. S4). The combination of the broad spectrum FLT3 inhibitor, PKC412, and CGM097 led to more killing of MOLM13 cells than either drug alone and was found to be nearly additive (ED50) to synergistic (ED75–ED90; Supplementary Fig. S5). We also tested the drug combination against MOLM14 cells and observed synergy (Fig. 4A). Similarly, the combination of CGM097 and a more highly selective inhibitor of FLT3, AC220, led to synergy against mutant FLT3–positive cells (Table 1; Supplementary Fig. S6). Synergy between FLT3 inhibition and CGM097 correlated with upregulation of expression levels of proapoptotic PUMA (Fig. 4E) and downregulation of antiapoptotic Myeloid cell leukemia-1 (Mcl-1; Fig. 4F).

Since coculturing AML cells with stroma leads to upregulation of antiapoptotic proteins (29), use of a p53 activator
(proapoptotic) should help to counteract this. This led us to explore the combination of CGM097 with either PKC412 or AC220 against mutant FLT3–positive cells cultured in the presence of 90% stromal-conditioned media (SCM) from the human stromal cell lines, HS-5 or HS27a. We observed HS-5–derived SCM or HS27a-derived SCM to confer partial protection to PKC412 or AC220-treated mutant FLT3–positive cells (Fig. 4D and Supplementary Fig. S6). Addition of CGM097 was able to counteract the SCM-induced protection from FLT3 inhibitors. In fact, the combination of CGM097 and PKC412 or CGM097 and AC220 was synergistic against mutant FLT3–positive cells cultured in the presence of SCM. This synergy was comparable with that observed for these drug combinations against mutant FLT3–positive cells cultured in the presence of RPMI + 10% FBS (Fig. 4B and C and Supplementary Fig. S6; Table 1).

Analysis of AML patient samples suggests that mutant RAS and mutant p53 are in general mutually exclusive (30). Consequently, we were interested in testing the ability of CGM097 to synergize with a downstream inhibitor of RAS signaling, presuming a combined effect of suppressing mutant RAS and inducing WT p53. NRAS mutant, p53 WT human primary AML sample (AML #10) showed a modest response to CGM097 alone. The modest response of the mutant RAS-expressing patient sample further prompted us to investigate the ability of RAS pathway inhibition and WT p53 activation to synergize. Similar to previous work that showed synergy between the MEK inhibitor AZD6244 (selumetinib) and nutlin (31), we also observed AZD6244 and CGM097 to synergistically inhibit proliferation and induce apoptosis in mutant NRAS-expressing OCI-AML3 cells (Fig. 5A–C and Supplementary Fig. S7).

CGM097 shows activity in vivo against WT p53-expressing AML.

To determine the activity of HDM2 inhibition in vivo, CGM097 was tested in a p53 WT AML patient–derived mouse xenograft (Fig. 6). Treatment of tumor bearing mice began when mice displayed substantial activity against WT p53-expressing AML.

Discussion

p53 protein levels are strictly regulated and are suppressed by a negative regulator of p53, the E3 ubiquitin protein ligase MDM2 (7). MDM2 interacts with p53 through direct binding to the p53 amino terminus; the binding of MDM2 to p53 makes p53 a target for ubiquitylation and consequent protein degradation (7). Small-molecule inhibitors and peptides have been developed to inhibit the association between HDM2 and p53 by targeting the p53 pocket of HDM2, thereby blocking the interaction between p53 and HDM2 and consequently stabilizing p53 such that its tumor suppressor activity is renewed (9, 33–37).

As p53 mutations in AML are rare and AML is in need of new and improved treatment strategies, this is an ideal disease target for small-molecule inhibitors of MDM2. Indeed, preclinical studies with nutlin-3 have shown activity in AML (10–12). Here, we introduce the novel HDM2 inhibitor, CGM097, which displayed substantial activity against WT p53-expressing AML.

Figure 3.
Selective targeting of p53 by CGM097. A and B, ELISA assay performed with anti-MIC for 15 minutes. Data for each sample were generated using supernatant collected from individual wells of a 24-well plate, with each well representing a single drug concentration.

1,000 1,500 2,000 2,500 3,000 3,500 4,000
0 500 250 100 200 300 400 500

**A**

**B**

<table>
<thead>
<tr>
<th>[CGM097, nmol/L]</th>
<th>[CGM097, nmol/L]</th>
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</thead>
<tbody>
<tr>
<td>0</td>
<td>500</td>
</tr>
<tr>
<td>250</td>
<td>1,200</td>
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<td>500</td>
<td>1,800</td>
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<table>
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<tr>
<th>MIC-1 secretion (% of control)</th>
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<tbody>
<tr>
<td>BM1</td>
</tr>
<tr>
<td>BM2</td>
</tr>
<tr>
<td>BM3</td>
</tr>
<tr>
<td>BM4</td>
</tr>
<tr>
<td>MOLM14</td>
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**Analysis of cell lines**

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>[CGM097, nmol/L]</th>
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<tbody>
<tr>
<td>HEL</td>
<td>1,800</td>
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<tr>
<td>OCI-AML3</td>
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**Analysis of patient sample**

<table>
<thead>
<tr>
<th>AML Sample</th>
<th>[CGM097, nmol/L]</th>
</tr>
</thead>
<tbody>
<tr>
<td>AML #10</td>
<td>1,200</td>
</tr>
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</table>

**Discussion**

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cell lines and selective toxicity toward primary AML cells expressing WT p53.

The synthesis and binding affinity to MDM2 of CGM097 have been described and disclosed (38). Briefly, a screen of 50,000 compounds exploiting a three hotspot 2D/3D pharmacophore led to identification of a series of isoquinolinones, the subsequent optimization of which revealed a novel binding mode with changes in protein organization associated with His 96 interactions (38). Additional changes resulted in the clinical candidate CGM097, a substituted 1,2-dihydroisoquinolinone derivative designed to mimic three key hydrophobic interactions made by p53 residues with Phe19, Trp23, and Leu26 in the HDM2 pocket (8, 39). CGM097 (structure revealed in 38, 40) binds to the p53 pocket of HDM2 with high potency and exceptional selectivity; in biochemical assays, CGM097 displaces the p53 peptide from the surface of HDM2 with an IC50 of 1.7 nmol/L, compared with an IC50 of 2,000 nmol/L for HDMX (38, 40). In a cell proliferation assay, CGM097 inhibited the growth of HCT-116 p53WT cells with a GI50 of 454 nmol/L and of HCT-116 p53-null cells with a GI50 of 15,983 nmol/L.

Table 1. Combination indices for studies testing the combination of either PKC412 + CGM097 or AC220 + CGM097 in the presence of RPMI + 10% FBS, HS-5 SCM (90%), or HS27a SCM (90%).

<table>
<thead>
<tr>
<th>Condition</th>
<th>ED50</th>
<th>ED25</th>
<th>ED75</th>
<th>ED90</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPMI + 10% FBS (PKC412 + CGM097)</td>
<td>0.76857</td>
<td>0.73627</td>
<td>0.69571</td>
<td>0.66305</td>
</tr>
<tr>
<td>HS-5 SCM (PKC412 + CGM097)</td>
<td>0.74431</td>
<td>0.63563</td>
<td>0.54300</td>
<td>0.46402</td>
</tr>
<tr>
<td>HS27a SCM (PKC412 + CGM097)</td>
<td>0.68059</td>
<td>0.60298</td>
<td>0.54904</td>
<td>0.51249</td>
</tr>
<tr>
<td>RPMI + 10% FBS (AC220 + CGM097)</td>
<td>0.80274</td>
<td>0.81577</td>
<td>0.86357</td>
<td>1.04731</td>
</tr>
<tr>
<td>HS-5 SCM (AC220 + CGM097)</td>
<td>0.70215</td>
<td>0.60298</td>
<td>0.54904</td>
<td>0.51249</td>
</tr>
<tr>
<td>HS27a SCM (AC220 + CGM097)</td>
<td>0.4372</td>
<td>0.57599</td>
<td>0.77412</td>
<td>1.04741</td>
</tr>
</tbody>
</table>

NOTE: All studies are representative of two independent studies for which similar results were observed.
nmol/L (38, 40). In contrast to HDM2 inhibitors, such as nutlin-3, which have been shown to display limited bioavailability in oral formulations in preclinical models (41), CGM097 is well absorbed with a moderate to high oral bioavailability ranging from 57% to 81% across multiple animal species tested (mouse, rat, dog and monkey; refs. 38, 42). In addition, CGM097 displays an excellent pharmacokinetic profile, low human intrinsic clearance, and exceptional ability to selectively inhibit p53/Mdm2 over p53/Mdm4 with negligible activity against p53-null cells (38, 40, 42). Finally, CGM097 demonstrates dose-dependent antitumor activity in human primary tumor xenografts in a p53-dependent manner (38, 40, 42).

Therapies aimed at restoring activation of p53 to replenish its tumor suppressive role in malignancies carry the risk of p53-induced apoptosis in normal cells. However, it has been shown that a level of p53 activation can potentially be achieved through tight regulation of its duration and strength that is not toxic to normal tissues but that still allows tumor suppression (43–48). In support of this, minimal toxicity in preclinical mouse models was observed with Roche HDM2 inhibitors due to their short cellular presence and transient p53 stimulation; however, a lower binding affinity of these drugs, which were optimized for human MDM2, toward mouse MDM2 could not be ruled out as being responsible for this lower toxicity (19, 38). Indeed, adverse reactions and modest efficacy were associated with clinical testing of the Roche HDM2 inhibitor, RG7112 (18). In addition, mutations conferring resistance to MDM2 inhibitors have been identified in preclinical studies (49–50). This suggests a need for identification and development of novel MDM2 inhibitors, as well as perhaps new MDM2 inhibitor–associated combination therapy approaches that may be more efficacious than MDM2 inhibition alone.

Figure 5.
Potentiation of effects of AZD6244 by CGM097 against mutant RAS-positive AML cells. A, approximately 3-day treatment of mutant NRAS-expressing OCI-AML3 cells with AZD6244, CGM097, or a combination of both. This study is representative of two independent studies for which similar results were observed. B, combination indices calculated for studies in A. C, effects of AZD6244 + CGM097 on induction of apoptosis, as compared with either agent alone, against OCI-AML3 cells. Bar graph shown in C is representative of apoptosis data shown in Supplementary Fig. S7.
CGM097 exhibits 8-fold greater affinity for MDM2 over Nutlin-3 (32). Importantly, as shown in this report, CGM097 displayed little toxicity toward normal bone marrow samples while potently and selectively inhibiting the proliferation and viability of WT p53-expressing primary AML patient cells and AML cell lines. In vivo testing using a primagraft model suggested high sensitivity of primary AML patient cells to CGM097 inhibition, as after only a week of consecutive dosing, there were 0% of hCD45⁺ cells in all mice (n = 7) compared with 18% at the start of treatment. At the end of the experiment (69 days after tumor implantation), untreated mice had 87% hCD45⁺ cells present in their blood, while the% hCD45⁺ cells in mice treated with CGM097 remained at 0%.

These encouraging preclinical results support the clinical testing of CGM097 in AML patients. Presently, CGM097 is being investigated in a phase I dose-escalation study in adult patients with selected advanced solid tumors expressing WT p53.

We also show the ability of CGM097 to synergize with selectively inhibitory TKIs, including inhibitors of mutant FLT3 and RAS-mediated signaling, even in the presence of secreted cytoprotective stroma–derived cytokines. These results suggest that CGM097 could potentially be used in combination with other targeted agents against oncogene-driven AML, as well as synergize in the presence of growth promoting factors and potentially override stromal-mediated drug resistance. Taken together, our findings support the further development of CGM097 as a potential novel therapeutic, alone or in combination with other targeted therapies for the treatment of AML.

Disclosure of Potential Conflicts of Interest

J.D. Griffin reports receiving commercial research grant from Novartis. No potential conflicts of interest were disclosed by the other authors.

Figure 6.

In vivo activity of CGM097 in p53 WT patient-derived AML xenograft. A, tumor growth curves of a patient-derived p53 WT AML xenograft treated with CGM097 or ARA-C (standard of care control). Treatment with CGM097 was initiated on day 35 postimplantation, when tumor burden reached 18% (measured by% hCD45⁺ cells), and continued until day 69 when tumor burden in the untreated group reached 87%. CGM097 significantly reduced in vivo tumor growth (P = 0.0004 by the two-tailed unpaired Student t test). Treatment with ARA-C was initiated on day 35 postimplantation, and stopped on day 50 due to body weight loss in all treated mice. Error bars, SEM. B, percent body weight change in mice following treatment. CGM097 dosed at 100 mg/kg per os as every day for 34 days was well tolerated with no significant body weight changes. ARA-C dosed at 50 mg/kg intraperitoneally every day for 15 days was not well tolerated and all mice were sacrificed on day 50 after tumor implantation. Error bars, SEM. AML model details: HAMLX5340: FLT3-ITD; NPM1 mut; p53WT.

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Acknowledgments

The authors thank Amanda L. Christie for her assistance with statistical analysis.

Grant Support

J.D. Griffin received NIH grant CA66996 and the work of all authors (with the exception of employees of Novartis Pharmaceuticals and M. Sattler) related to CGM097 was supported by this grant. M. Sattler received NIH grant CA134660-03.

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Received May 26, 2015; revised July 2, 2015; accepted July 19, 2015; published OnlineFirst July 23, 2015.

www.aacrjournals.org Mol Cancer Ther; 14(10) October 2015 2257

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References

16. Martins CP, Brown-Swigart L, Evan GI. Modeling the therapeutic eff

Published OnlineFirst July 23, 2015; DOI: 10.1158/1535-7163.MCT-15-0429


Molecular Cancer Therapeutics

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