IDENTIFICATION AND CHARACTERIZATION OF THE FIRST SMALL-MOLECULE INHIBITOR OF MDMX

*Damon Reed¹, *Ying Shen¹, Anang Shelat², Alexander Arnold², Antonio Ferreira³, Fangyi Zhu², Nicholas Mills², David Smithson², Catherine Regni², Don Bashford³, Samantha Cicero¹, Brenda Schulman³, Aart G. Jochemsen⁴, Kiplin Guy², and Michael A. Dyer ¹

From Department of Developmental Neurobiology¹, Chemical Biology and Therapeutics² and Structural Biology³,
St. Jude Children’s Research Hospital, Memphis, TN 38105
From Department of Molecular and Cellular Biology⁴,
Leiden University Medical Center, Leiden, Netherlands

Address correspondence to: Michael A. Dyer, MS 323, St. Jude Children’s Research Hospital, 262 Danny Thomas Place, Memphis, TN 38105, USA. Phone: 901-595-2257; FAX: 901-595-3143; e-mail: michael.dyer@stjude.org

*These authors contributed equally to the work.

The p53 pathway is disrupted in virtually every human tumor. In approximately 50% of human cancers, the p53 gene is mutated, and in the remaining cancers, the pathway is deregulated by genetic lesions in other genes that modulate the p53 pathway. One common mechanism for inactivation of the p53 pathway in tumors that express wild-type p53 is increased expression of MDM2 or MDMX. MDM2 and MDMX bind p53 and inhibit its function by distinct nonredundant mechanisms. Small-molecule inhibitors and small peptides have been developed that bind MDM2 in the p53-binding pocket and displace the p53 protein, leading to p53-mediated cell cycle exit and apoptosis. To date, peptide inhibitors of MDMX have been developed, but no small-molecule inhibitors have been reported. We have developed biochemical and cell-based assays for high-throughput screening of chemical libraries to identify MDMX inhibitors and identified the first MDMX inhibitor, SJ-172550. This compound binds reversibly to MDMX and effectively kills retinoblastoma cells in which the expression of MDMX is amplified. The effect of SJ-172550 is additive when combined with an MDM2 inhibitor. Results from a series of biochemical and structural modeling studies suggest that SJ-172550 binds the p53-binding pocket of MDMX, thereby displacing p53. This lead compound is a useful chemical scaffold for further optimization of MDMX inhibitors that may eventually be used to treat pediatric cancers and various adult tumors that overexpress MDMX or have similar genetic lesions. When combined with selective MDM2 inhibitors, SJ-172550 may also be useful for treating tumors that express wild-type p53.

Tumorigenesis is a multistep process that involves deregulation of several pathways that are crucial for cell growth and survival (1). The p53 pathway regulates cell survival in response to cellular stress (e.g., DNA damage) or oncogenic stress (e.g., Rb pathway deregulation) (2,3) and is suppressed in virtually every human cancer by genetic lesions in the p53 gene or other
components of the pathway (4). Approximately half of all cancers express wild-type p53, and considerable research over the past decade has focused on inducing p53-mediated cell death in these tumors (4,5). Most efforts to date have focused on inhibiting MDM2, a negative regulator of p53 (6-14).

Another key regulator of the p53 pathway is a protein related to MDM2 called MDMX (15-17). MDM2 and MDMX share homology in their p53-binding domains, but MDMX is believed to regulate p53 through distinct mechanisms. Specifically, MDM2 primarily regulates p53 stability and subcellular localization, while MDMX may directly regulate p53 transcription (17-21). MDMX is genetically amplified in 19% of breast carcinomas, 19% of colon carcinomas, 18% of lung carcinomas, and a smaller percentage of gliomas (17). One of the best-characterized tumors with an MDMX amplification is retinoblastoma. Approximately 65% of human retinoblastomas have increased MDMX copy number, which correlates with increased MDMX mRNA and protein (22). Previous studies have demonstrated that the MDMX amplification suppresses p53-mediated cell death in Rb pathway–deficient retinoblasts (22).

A general consensus is emerging that to efficiently induce a p53 response in tumor cells that express wild-type p53, it may be necessary to inactivate both MDM2 and MDMX (18,23,24). To date, no screens to identify small-molecule inhibitors of MDMX have been reported, and MDM2 inhibitors probably do not bind as efficiently to MDMX due to structural differences in the p53-binding pockets of the 2 proteins (25-27). Consistent with this theory, nutlin-3a binds MDMX with at least a 40-fold weaker equilibrium binding constant (K_d) than for MDM2 (22).

Therefore, to identify small molecules that bind MDMX and prevent its interaction with p53, we developed biochemical and cell-based assays suitable for high-throughput screening (HTS) of chemical libraries. Using this approach, we have identified the first MDMX inhibitor, SJ-172550, and demonstrated that it can efficiently kill MDMX-amplified retinoblastoma cells. SJ-172550 functions in an additive manner with the MDM2 inhibitor nutlin-3a, thereby confirming the importance of targeting both of these negative regulators of p53 in cancer cells. This validated MDMX inhibitor provides a valuable lead compound and chemical scaffold for further chemical modification to develop a high-affinity MDMX inhibitor with good bioavailability, pharmacokinetics and pharmacodynamics.

**Experimental Procedures**

**Plasmid constructs and protein production.** The p53-binding domain of mouse and human MDMX (a.a. 1-185) and human MDM2 (a.a. 1-188) were amplified by PCR and cloned into the pGEX-4T1 plasmid. Recombinant GST-fusion proteins were prepared in BL21(DE3) E. coli cells. The lysates were cleared by spinning at 100,000 ×g, and the supernatant was loaded onto a 5-ml GSTrap Fast-Flow column (GE Life Sciences). Subsequent purification included a Mono-Q column and a S200 gel filtration column. Peak fractions were combined and dialyzed against PBS (pH 7.6) containing 2 mM PMSF.

**Fluorescence polarization assays.** Fluorescence polarization (FP) assays were conducted in assay buffer containing 10 mM Tris (pH 8.0), 42.5 mM NaCl, and 0.0125% Tween-20. The wild type p53 peptide (a.a. 15-29) was GSGSSQETFSDLWKLLPEN and the mutant AAA-p53 peptide was GSGSSQETFADLALKAPEN. The FP
assays were carried out using 2.5 nM FITC-peptide (or 15 nM Texas Red) and 1 µM MDM2-GST or MDMX-GST. For MDM2-p53 or MDMX-p53 inhibitor assays, small molecules were preincubated with the recombinant protein for 30 min. The labeled peptide was then added and incubated for 45 min. FP assays were conducted in 384-well black microplates (Corning Life Sciences). The FP FITC assays were analyzed using an EnVision Multilabel plate reader with a 480-nm excitation filter, a 535-nm static and polarized filter, and an FP FITC dichroic mirror. The unlabeled competitor peptide and nutlin-3 were used as positive controls, and the alanine-substituted p53 peptide (AAA-p53) was used as a negative control. To minimize the possibility of false-positive caused by endogenous fluorescence from the compounds in the library, we also developed an FP assay with the Texas Red fluorophore. This assay was the same as that used for the FITC experiments, except the FP Texas Red assays were run using a 555-nm excitation filter, a 632-nm static and polarized filter, and an FP Texas Red FP dichroic mirror.

Chemical library and high-throughput screening. The screening library consisted of 296,488 unique compounds (ChemDiv, ChemBridge, and Life Chemicals) arrayed individually at 10 mM in DMSO in 384-well polypropylene plates. The quality of compounds was assured by the vendor as 90% pure. HTS was carried out on a system developed by High Resolution Engineering with integrated plate incubators (Liconic). Plates were transferred from instrument to instrument by a Staübl T60 robot arm. Assay materials were dispensed in bulk by using Matrix Wellmates (Matrix Technologies). Compound plates were centrifuged in a Vspin plate centrifuge (Velocity11). All compound transfers were accomplished by using a 384-well pin tool with 10-nL hydrophobic surface–coated pins (V & P Scientific). These pins allowed for the delivery of 25 nL to achieve a final compound concentration of 10 µM. The fluorescent signal was measured using an EnVision multilabel plate reader.

RESULTS

Characterization of an MDMX-p53 binding assay for high-throughput screening. To identify MDMX inhibitors by HTS of chemical libraries, we developed an FP assay (28) to detect the binding of the p53 peptide to GST-DMMX1-185 in 384-well plates. This assay is based on the retention of polarization during fluorescence spectroscopy of the p53 peptide conjugated to a fluorophore such as FITC (Fig. 1A). FP is inversely proportional to the rotational diffusion of the fluorophore, and for our experiments, it was measured using an FP spectrometer. The polarization of free p53-FITC peptide (2.5 nM) was 100 mP, and with increasing concentrations of purified GST-DMMX1-185 protein (Sup. Fig. 1), the polarization peaked (100%) at 280 mP (Fig. 1B). Similar data were obtained using GST-DMM21-188 (Fig. 1B, Sup. Fig. 1). From these curves, the EC50 for p53 peptide–MDMX was 0.36 µM, and that for p53 peptide–MDM2 was 0.23 µM (Fig. 1B). Biacore experiments provided similar binding constants for GST-DMMX1-185 (Kd=1.05 µM) and GST-DMM21-188 (Kd=1.03µM) (Sup. Fig. 1). Isothermal titration calorimetry (ITC) measurements using a purified minimal p53-binding domain of MDMX23-111 gave similar binding constants (Sup. Fig. 1).

To test the specificity of our FP assay for MdmX/MDM2 binding to p53, we performed a competition experiment with unlabeled p53 peptide and a version of the AAA-p53 peptide that is defective for binding to MDM2/MDMX (29). The protein concentration in this assay was 1 µM, and the p53-FITC peptide
concentration was 2.5 nM. The EC\textsubscript{50} from this experiment was 0.42 µM for GST-MDMX\textsuperscript{1-185} and 0.30 µM for GST-MDM2\textsuperscript{1-188} (Fig. 1C). AAA-p53 showed no evidence of competition at any concentration tested (0.5-200 µM) (Fig. 1C).

To test whether our FP assay was suitable for identifying small-molecule inhibitors of MDMX-p53 binding, we performed a dose-response experiment using nutlin-3a at concentrations ranging from 0.5 nM to 300 µM (Fig. 1D). Nutlin-3a was originally identified as an MDM2 inhibitor (6), but it also binds specifically to MDMX, albeit with a much weaker K\textsubscript{d} (22). The protein concentration was held constant at 1 µM, and the peptide concentration was 2.5 nM for each concentration of nutlin-3a tested (Fig. 1D). The EC\textsubscript{50} for binding of nutlin-3a to MDM2 was 0.28 µM, and that to MDMX was 20.1 µM (Fig. 1D).

**High-throughput screening of a chemical library to identify novel MDMX inhibitors.** To identify novel MDMX inhibitors, we performed an HTS of the St. Jude chemical library (296,488 unique compounds) by using the GST-MdmX\textsuperscript{1-185}/p53-FITC peptide FP assay (Fig. 1E). A total of 357,120 wells (compounds and controls) were screened over the course of 13 days by using 930 plates. We selected the mouse MdmX protein for the HTS, because expression of the recombinant protein in \textit{E. coli} was more efficient than human MDMX. Compounds were screened at a final concentration of 10 µM. The z', z-factor, and average endpoint map analysis illustrated that the biochemical assay was sufficiently stable throughout the day, on each day of the run. The assay was also reproducible day-to-day, over the course of the entire screen (Sup. Fig. 2). Each plate had a series of positive and negative controls, and these were well separated over the 13-day screen (Fig. 1E).

To reduce the probability of selecting false-positive compounds from the primary screen, we performed a receiver operating characteristic (ROC) analysis using different activity thresholds for compound selection. This analysis led us to select the 70% activity threshold to obtain 4,363 compounds for subsequent validation. To eliminate compounds with intrinsic fluorescence emission spectra that overlapped with FITC, we performed a secondary FP assay using a Texas Red–conjugated peptide. This analysis was performed on all 4,363 compounds that met the 70% activity cutoff (10 µM) in triplicate. The top 1,000 compounds were then selected from the Texas Red FP assay based on their percentage inhibition, and an additional 152 compounds were included (total 1,152) to provide sufficient coverage of chemical scaffolds to begin to establish structure-activity relationships for our candidate MDMX inhibitors (Fig. 2A).

**Analysis of active compounds from MDMX inhibitor high-throughput screening.** To further characterize the 1,152 active compounds, we measured each compound’s binding constant to MdmX (mouse), MDMX (human), and MDM2 (human) by performing a dose-response assay in triplicate (Fig. 2A-C). There was no significant difference in the proportion of validated hits between 70% and 100% activity using the FITC FP assay data (compare gray to black bars, Fig. 2B). In contrast, when the 1,152 compounds were screened in triplicate using the Texas-Red FP assay, the percent of validated hits was more broadly distributed across the activity range for the assay (light blue bars, Fig. 2B). Therefore, the Texas-Red FP data were strongly predictive of validated hits (dark blue bars, Fig. 2B) and provided better separation for the validated subset of compounds that gave a dose response.
To complement these biochemical studies, we developed a cell-based assay to further characterize the activity of the 1,152 compounds on retinoblastoma cells that have an *MDMX* amplification (Weri1) or a cell line that is p53-deficient (SJmRbl-8) (22). CellTiter-Glo assay was used to measure intracellular ATP levels as an indicator of viability. SJmRbl-8 cells and Weri1 cells showed a linear relationship between luminescence and cell number (Sup. Fig. 3A, B). As a positive control for cytotoxicity, we used vincristine, which is a nonselective microtubule inhibitor that disrupts chromosome segregation during mitosis and kills both cell lines with similar EC$_{50}$ values (Sup. Fig. 3C). As a positive control for p53-selective cytotoxicity, we used nutlin-3a, which selectively kills Weri1 cells with an *MDMX* amplification and is less cytotoxic against p53-deficient SJmRbl-8 cells (Sup. Fig. 3D). We also used the BJ cells, an hTERT-immortalized human foreskin fibroblast cell line, as an additional control to estimate general cytotoxicity of the compounds in our lead compound collection. We carried out a dose-response cytotoxicity assay for each cell line on all 1,152 compounds in triplicate. Overall, the assay performed well in this HTS format, and we identified a series of compounds from our active compounds set with significant selective cytotoxicity against the retinoblastoma cells (Sup. Fig. 3E, F).

To integrate and visualize the dose-response data from the biochemical assays of *MDMX* and MDM2 and the cell-based data and chemical scaffolds represented in the 1,152 compounds, we overlaid biochemical and cell-based data onto a network graph constructed to represent related families of chemotypes within the lead compound set (Sup. Fig. 4). This approach allowed us to quickly identify scaffolds with the desired profiles showing strong binding to MDMX and cytotoxicity against an MDMX-amplified retinoblastoma cell line. On the basis of these data, we selected 11 representative compounds from clusters 1, 4, 5, 7, 8, 11, and 54 for further analysis (Fig. 2D-J, Sup. Fig. 4, and Sup. Table 1). Several clusters that looked promising based on the aforementioned criteria were eliminated from further analysis due to their containing potentially problematic chemical functionalities such as unsubstituted quinones, maleimides and other reactive acylators, compounds capable of Michael addition; as well as benzothioates, and other compounds with predicted redox activity in cells (Sup. Fig. 4).

**SJ-134433 and SJ-044557 covalently modify the MDMX protein.** Among the 11 compounds selected for further analysis, SJ-134433 and SJ-044557 had excellent profiles (Sup. Fig. 6) with good binding constants for MDMX, some selectivity for MDMX over MDM2, and efficient killing of retinoblastoma cells with selectivity for the Weri1 line (Sup. Table 1). To begin characterizing these compounds, we performed an isothermal denaturation assay and a redox assay on SJ-134433, SJ-044557, and the other 9 compounds (Sup. Fig. 5 and Sup. Table 1). The rationale underlying the isothermal denaturation assay is that binding of a small molecule to the p53-binding pocket of MDMX may stabilize the protein, and in the presence of the SYPRO orange hydrophobic dye, the temperature for denaturation and dye binding would shift (30,31). Indeed, GST-MDMX$^{1-185}$ showed a melting point shift in the presence of p53 peptide from 46.9±0.6 °C for native GST-MDMX$^{1-185}$ protein to 50.8±0.6 °C for GST-MDMX$^{1-185}$ protein bound to p53 peptide (Sup. Fig. 5A, B). However, neither SJ-044557 nor SJ-134433 exhibited a thermal shift; in fact, it appeared that the protein was destabilized in this assay (Sup. Fig. 5).
To explore the possibility that these 2 compounds exhibited redox activity, we performed an assay to detect compounds capable of reducing resazurin to resorufin, a redox couple relevant to oxygen tension in mammalian cells (32). DMSO was used as a negative control, and a pyrimidotirazinedione-containing compound was used as the positive control. SJ-044557 and SJ-134433 showed some redox activity (Sup. Table 1). Next, we explored the stability of the compounds in our FP buffer to determine if they were unstable and if any of the degradation products were reactive species that covalently modified the MDMX protein and blocked p53 binding. Both compounds were unstable after 24 h, and SJ-134433 degraded after 2 h in FP buffer (Sup. Fig. 6). To directly determine whether the purified MDMX23-111 protein was covalently modified by either compound, we performed high-resolution mass spectrometry following incubation with SJ-044557 or SJ-134433. Both showed a shift in their mass consistent with covalent modifications (Sup. Fig. 6). While these inhibitors might prove useful as tools for interrogating MDMX function, they are not suitable for further development and were thus abandoned.

**SJ-172550 is stable and reversibly binds MDMX to inhibit p53 binding.** A more detailed analysis of the 11 selected compounds revealed that SJ-172550 had an excellent chemical profile, with respect to chemical stability, thermal stability, redox potential, solubility, and permeability. Unlike SJ-134433 and SJ-044557, SJ-172550 was stable in solution (Fig. 3B, C) and did not modify the MDMX protein by covalent binding in our FP assay buffer (Fig. 3D). Moreover, the compound exhibited strong thermal stabilization (Sup. Fig. 5) and had undetectable redox activity (Fig. 3A and Sup. Table 1). To confirm that SJ-172550 binds MDMX reversibly, we incubated the compound with MDMX for 2 h in FP buffer and then removed the compound by dialysis. As a positive control, we used the p53 peptide and nutlin-3a. Our results showed that SJ-172550 bound MDMX reversibly (Fig. 3E), unlike SJ-044557 or SJ-172550 (Fig. 3F).

**SJ-172550 inhibits MDMX-p53 binding in cultured cells.** To test whether SJ-172550 and nutlin-3a have additive or synergistic effects on retinoblastoma cells, we performed an isobologram experiment with the 2 compounds. The data suggested that nutlin-3a and SJ-172550 act in an additive manner to kill MDMX-amplified human retinoblastoma cells (Fig. 4A). Next, we exposed Weri1 and RB355 retinoblastoma cells and ML-1 leukemia cells (with wild type p53) to SJ-172550 (20 µM) for 20 h and analyzed the p53 and activated Caspase-3 levels by immunoblotting and immunofluorescence to study the mechanism of cell death that occurred. As positive controls, we exposed Weri1 cells to nutlin-3a (5 µM) for 20 h or 5 Gy IR. For a negative control, we used DMSO. As expected, the Weri1 cells exposed to nutlin-3a or IR showed a robust accumulation of p53 (Fig. 4B, D). In contrast, the cells exposed to SJ-172550 did not exhibit the same level of accumulation of p53 (Fig. 4B, D). This is consistent MDMX’s role in regulating transcriptional activation of p53-responsive promoters but not p53 protein levels. Apoptosis was robustly induced after exposure to SJ-172550 (Fig. 4C, E) and cells exited the cell cycle (Fig. 4F). Real-time RT-PCR and immunoblotting analysis of these cells revealed that there was also an induction of p53 target genes, but it was not as robust as that observed with nutlin-3a or IR (Fig. 4G,H). More importantly, the cell death mediated by SJ-172550 was p53-dependent (Fig. 4I-K). In addition, HCT116 cells were sensitive to SJ-172550 but p53-
deficient HCT-116 cells were not (Sup. Fig. 8). SJSA-X cells expressing high levels of MDMX were also sensitive to SJ-172550 (Sup. Fig. 8).

To determine whether SJ-172550 disrupts the MDMX-p53 interaction in cells in culture, we performed coimmunoprecipitation (co-IP) experiments in the presence of the compound. Reciprocal co-IP experiments with antibodies against MDMX and p53 in C33A (human cervical carcinoma) cells demonstrated partial inhibition of MDMX-p53 binding in cells (Sup. Fig. 7). Similar data were obtained using HER (human embryonic retina) cells and Weri1 retinoblastoma cells (Sup. Fig. 7). Together, these results suggest that the p53-MDMX interaction was at least partially inhibited by SJ-172550, despite its relatively low cell permeability (see Sup. Table 1).

**Computational model of SJ-172550 binding to MDMX.** X-ray crystallographic studies have provided high-resolution structures of MDM2 and MDMX bound to p53 (Sup. Fig. 9A) (26,27,33,34). We overlaid these 2 structures and determined that the Cα root mean square deviation (RMSD) was 3.9 Å, suggesting that although the overall fold was well conserved, the tertiary structures of MDM2 or MDMX bound to p53 are significantly different. This can be more readily visualized using a space-filling representation of the overlaid structures. In particular, the structure of the p53-binding pocket of MDMX was smaller than that of MDM2 (Sup. Fig. 9B). When nutlin-3a was bound to MDM2, the tertiary structure of the pocket underwent a small change (Cα RMSD = 0.82 Å) (Sup. Fig. 9C). When the structure of MDM2 bound to nutlin-3a was overlaid with that of MDMX bound to p53, the smaller binding pocket of MDMX may explain the lower affinity binding of nutlin-3a to MDMX, as compared to that of MDM2 (Sup. Fig. 9D, E).

We used both AutoDock 4.2(35) and the fastdock algorithm in Scigress Explorer v7.7 to model the binding of nutlin-3a to MDM2 and found excellent agreement between the computational model and the native conformation reported in previous cocrystallization studies (Sup. Fig. 9F) (6). Using the same computational approach, the binding of SJ-172550 to the p53-binding pocket of MDMX was modeled, yielding two important structures (Fig. 5A-D and Sup. Fig. 9G). Together, these results provide a plausible mechanism of action for SJ-172550—SJ-172550 may occlude the p53-binding pocket of MDMX, thereby inhibiting p53 binding and activity. To test this directly, we generated a series of 7 mutants in the MDMX binding pocket and purified the protein for binding studies (Fig. 5E-G, Sup. Fig. 9H,I and Supplemental Information). Some of the mutants (e.g. H54F) were predicted to displace SJ-172550 without affecting peptide binding and other mutants (e.g. M53L) were predicted to make the binding pocket of MDMX more like MDM2 and thereby reduce binding of SJ-172550 and increase binding of nutlin-3a without affecting peptide binding. These data provide additional validation for our proposed mechanism of SJ-172550 inhibition of the p53-MDMX interaction.

**DISCUSSION**

Although several small-molecule inhibitors of MDM2 have been identified (6,14), this is the first report to identify a small-molecule inhibitor of MDMX. There is growing evidence that MDM2 and MDMX inhibit p53 through distinct mechanisms and that simultaneous inhibition of these 2 proteins in tumor cells that express wild-type p53 may be more effective at killing the cells than the
inhibition of MDM2 alone. Our results presented here complement important previous studies on high-affinity peptide inhibitors of MDMX and MDM2 (9,34). MDMX inhibitors alone (peptide or small-molecule) may be useful for treating tumors such as retinoblastoma that show increased MDMX expression (22). In addition, they may be effective when combined with MDM2 inhibitors to induce a robust p53 response in cancer cells that express wild-type p53 (36).

We have developed and optimized a biochemical assay for HTS of MDMX inhibitors. We screened a diverse chemical library and identified compound SJ-172550 as the first small-molecule inhibitor of MDMX with a low micromolar binding constant. SJ-172550 reduced p53 binding in vitro and had little or no redox activity. It also did not covalently modify MDMX, but rather thermostabilized the protein and reversibly bound it, which was consistent with our modeling of SJ-172550 binding to the p53-binding pocket of MDMX. When retinoblastoma cells expressing wild-type p53 and high levels of MDMX were exposed to SJ-172550 in vitro, they showed evidence of p53-mediated cytotoxicity. More importantly, the death was p53 dependent because an siRNA to p53 prevented SJ-172550-mediated cell death. While these data point to a p53-dependent cell death mechanism, they do not rule out the possibility of off-target binding of SJ-172550. Indeed, many lead compounds and drugs show off-target effects.

In combination with the MDM2 inhibitor nutlin-3a, SJ-172550 showed additive cytotoxicity in cells that expressed wild-type p53. Thus, we propose that SJ-172550 binds the p53-binding pocket of MDMX, thereby freeing p53 to induce apoptosis. This compound represents a bona fide lead and together with the other promising lead scaffolds from this study, can now be used for further medicinal chemical analyses including optimization of affinity, specificity, and cell permeability and assessment of pharmacokinetics and toxicity.

It has been shown that MDM2 and MDMX can form a heterodimer through their Ring domains and this may regulate MDM2-mediated degradation of p53 (37-39). We found that the overall p53 protein levels are not dramatically altered following exposure of cultured cells to SJ-172550. The identification of the first small molecule inhibitor will allow researchers to probe this mechanism further by comparing the effect of MDMX protein loss to inhibition of MDMX-p53 binding on p53 stability.

One of our key findings from the analysis of biochemical and cell-based assay data was that the compounds that had the best binding constants for MDMX were not necessarily the ones that were most suitable for follow-up. For example, SJ-134433 had a very good binding constant for MDMX, but further analysis showed it was unstable in FP buffer, did not thermostabilize MDMX, had significant redox activity, and covalently modified the protein. These data clearly emphasize the importance of performing comprehensive characterization of active compounds to rule-out nonspecific mechanisms of action that make compounds unsuitable for further development. They also emphasize the need to select candidates for further work based on a balance of chemical and biological properties, rather than purely on potency or biochemical mechanism of action.

We have not fully validated the mechanism of action of SJ-172550, but it seems probable based upon our modeling and data that it binds the p53-binding pocket of MDMX and frees p53 to activate its target genes leading to cell cycle exit and apoptosis. Consistent with this model, we observed moderate p53-pathway activation...
in MDMX-amplified retinoblastoma cells and partial disruption of the MDMX-p53 interaction in cell lysates from retinoblastoma cells and other cell lines. We do not believe that the mechanism of action is through p53 protein stabilization based on immunoblotting and single cell immunostaining. A structural alignment of the binding pockets of MDMX and MDM2 was produced using the backbone atoms and provides some clues about where SJ-172550 may bind MDMX to induce p53-pathway activation. Mutations generated in the p53 binding pocket of MDMX provided additional support for this proposed mechanism. Additional x-ray crystallography and other structural studies are required to definitively show that SJ-172550 binds to the p53-binding pocket of MDMX. Nonetheless, this is the first small molecule MDMX inhibitor that has been identified with a low micromolar binding constant. It is important to note that SJ-172550 also binds MDM2, although less effectively. Our compound is clearly less effective against MDM2 than nutlin-3a (6) or other MDM2 inhibitors (14), and it is impossible to predict whether further refinement of MDMX binding will similarly improve MDM2 binding or lead to a high-affinity MDMX-selective inhibitor.
REFERENCES

1. Hanahan, D., and Weinberg, R. A. (2000) Cell 100, 57-70
2. Dang, J., Kuo, M. L., Eischen, C. M., Stepanova, L., Sherr, C. J., and Roussel, M. F. (2002) Cancer Res 62, 1222-1230
3. Vogelstein, B., Lane, D., and Levine, A. J. (2000) Nature 408, 307-310
4. Vousden, K. H., and Lu, X. (2002) Nat Rev Cancer 2, 594-604
5. Levine, E. M., Passini, M., Hitchcock, P. F., Glasgow, E., and Schechter, N. (1997) J Comp Neurol 387, 439-448.
6. Vassilev, L. T., Vu, B. T., Graves, B., Carvajal, D., Podlaski, F., Filipovic, Z., Kong, N., Kammlott, U., Lukacs, C., Klein, C., Fotouhi, N., and Liu, E. A. (2004) Science 303, 844-848
7. Marine, J. E. (2006) Heart Rhythm 3, 342-344
8. Anderson, J. J., Challen, C., Atkins, H., Suaeyun, R., Crosier, S., and Lunec, J. (2007) Int J Oncol 31, 545-555
9. Hu, B., Gilkes, D. M., and Chen, J. (2007) Cancer Res 67, 8810-8817
10. Udayakumar, T. S., Hachem, P., Ahmed, M. M., Agrawal, S., and Pollack, A. (2008) Mol Cancer Res 6, 1742-1754
11. Tovar, C., Rosinski, J., Filipovic, Z., Higgins, B., Kolinsky, K., Hilton, H., Zhao, X., Vu, B. T., Qing, W., Packman, K., Myklebost, O., Heimbrook, D. C., and Vassilev, L. T. (2006) Proc Natl Acad Sci U S A 103, 1888-1893
12. Coll-Mulet, L., Iglesias-Serret, D., Santidrian, A. F., Cosiaills, A. M., de Frias, M., Castano, E., Campas, C., Barragan, M., de Sevilla, A. F., Domingo, A., Vassilev, L. T., Pons, G., and Gil, J. (2006) Blood 107, 4109-4114
13. Kojima, K., Konopleva, M., Samudio, I. J., Shikami, M., Cabreira-Hansen, M., McQueen, T., Ruvolo, V., Tsao, T., Zeng, Z., Vassilev, L. T., and Andreeff, M. (2005) Blood 106, 3150-3159
14. Shangary, S., Qin, D., McEachern, D., Liu, M., Miller, R. S., Qiu, S., Nikolovska-Coleska, Z., Ding, K., Wang, G., Chen, J., Bernard, D., Zhang, J., Lu, Y., Gu, Q., Shah, R. B., Pienta, K. J., Ling, X., Kang, S., Guo, M., Sun, Y., Yang, D., and Wang, S. (2008) Proc Natl Acad Sci U S A 105, 3933-3938
15. Bartel, F., Schulz, J., Bohnke, A., Blumke, K., Kappler, M., Bache, M., Schmidt, H., Wurl, P., Taubert, H., and Hauptmann, S. (2005) Int J Cancer 117, 469-475
16. Shvarts, A., Bazuine, M., Dekker, P., Ramos, Y. F., Steegenga, W. T., Merckx, G., van Ham, R. C., van der Houven van Oordt, W., van der Eb, A. J., and Jochemsen, A. G. (1997) Genomics 43, 34-42
17. Danovi, D., Meulmeester, E., Pasini, D., Migliorini, D., Capra, M., Frenk, R., de Graaf, P., Francoz, S., Gasparini, P., Gobbi, A., Helin, K., Pelicci, P. G., Jochemsen, A. G., and Marine, J. C. (2004) Mol Cell Biol 24, 5835-5843
18. Toledo, F., Krummel, K. A., Lee, C. J., Liu, C. W., Rodewald, L. W., Tang, M., and Wahl, G. M. (2006) Cancer Cell 9, 273-285
19. Marine, J. C., Dyer, M. A., and Jochemsen, A. G. (2007) J Cell Sci 120, 371-378
20. Marine, J. C., and Jochemsen, A. G. (2004) Cell Cycle 3, 900-904
21. Migliorini, D., Lazzerini Denchi, E., Danovi, D., Jochemsen, A., Capillo, M., Gobbi, A., Helin, K., Pelicci, P. G., and Marine, J. C. (2002) Mol Cell Biol 22, 5527-5538
22. Laurie, N. A., Donovan, S. L., Shih, C. S., Zhang, J., Mills, N., Fuller, C., Teunisse, A., Lam, S., Ramos, Y., Mohan, A., Johnson, D., Wilson, M., Rodriguez-Galindo, C., Quarto, M., Francoz, S., Mendrysa, S. M., Guy, R. K., Marine, J. C., Jochemsen, A. G., and Dyer, M. A. (2006) *Nature* 444, 61-66

23. Hu, B., Gilkes, D. M., Farooqi, B., Sebti, S. M., and Chen, J. (2006) *J Biol Chem* 281, 33030-33035

24. Gu, J., Kawai, H., Nie, L., Kitao, H., Wiederschain, D., Jochemsen, A. G., Parant, J., Lozano, G., and Yuan, Z. M. (2002) *J Biol Chem* 277, 19251-19254

25. Bottger, V., Bottger, A., Garcia-Echeverria, C., Ramos, Y. F., van der Eb, A. J., Jochemsen, A. G., and Lane, D. P. (1999) *Oncogene* 18, 189-199

26. Popowicz, G. M., Czarna, A., Rothweiler, U., Szwagierczak, A., Krajewski, M., Weber, L., and Holak, T. A. (2007) *Cell Cycle* 6, 2386-2392

27. Popowicz, G. M., Czarna, A., and Holak, T. A. (2008) *Cell Cycle* 7, 2441-2443

28. Stricher, F., Martin, L., Barthe, P., Pogenberg, V., Mechulam, A., Menez, A., Roumestand, C., Veas, F., Royer, C., and Vita, C. (2005) *Biochem J* 390, 29-39

29. Lu, F., Chi, S. W., Kim, D. H., Han, K. H., Kuntz, I. D., and Guy, R. K. (2006) *J Comb Chem* 8, 315-325

30. Senisterra, G. A., and Finerty, P. J., Jr. (2009) *Mol Biosyst* 5, 217-223

31. Senisterra, G. A., Soo Hong, B., Park, H. W., and Vedadi, M. (2008) *J Biomol Screen* 13, 337-342

32. Lor, L. A., Schneck, J., McNulty, D. E., Diaz, E., Brandt, M., Thrall, S. H., and Schwartz, B. (2007) *J Biomol Screen* 12, 881-890

33. Kallen, J., Goepfert, A., Bleichschmidt, A., Izaac, A., Geiser, M., Tavares, G., Ramage, P., Furet, P., Masuya, K., and Lisztwan, J. (2009) *J Biol Chem* 284, 8812-8821

34. Pazgier, M., Liu, M., Zou, G., Yuan, W., Li, C., Li, J., Monbo, J., Zella, D., Tarasov, S. G., and Lu, W. (2009) *Proc Natl Acad Sci U S A* 106, 4665-4670

35. Morris, G. M., Huey, R., Lindstrom, W., Sanner, M. F., Belew, R. K., Goodsell, D. S., and Olson, A. J. (2009) *J Comput Chem* 30, 2785-2791

36. Laurie, N. A., Shih, C. S., and Dyer, M. A. (2007) *Curr Cancer Drug Targets* 7, 689-695

37. Stad, R., Little, N. A., Xiromimas, D. P., Frenk, R., van der Eb, A. J., Lane, D. P., Saville, M. K., and Jochemsen, A. G. (2001) *EMBO Rep* 2, 1029-1034

38. Linke, K., Mace, P. D., Smith, C. A., Vaux, D. L., Silke, J., and Day, C. L. (2008) *Cell Death Differ* 15, 841-848

39. Jackson, M. W., and Berberich, S. J. (2000) *Mol Cell Biol* 20, 1001-1007

**FOOTNOTES**

We would like to thank Taosheng Chen, Jimmy Chu and David Bouck for assistance with high throughput screening at the St. Jude High Throughput Screening Center. We thank David Miller for assistance with protein preparation. We thank Erin H. Seeley, Jamie L. Allen, and Richard M. Caprioli (all of the Mass Spectrometry Research Center, Vanderbilt Medical Center, Nashville, TN) for assistance with high-resolution MALDI mass spectrometry analyses. We also thank Brett Waddell for assistance with Biacore analysis and Angie McArthur for editing the manuscript. This work was supported by grants (to M.A.D.) from the National Institutes of
Health, Cancer Center Support from the National Cancer Institute, the American Cancer Society, Research to Prevent Blindness, the Pearle Vision Foundation, the International Retinal Research Foundation, the Pew Charitable Trust, and the American Lebanese Syrian Associated Charities (ALSAC).

FIGURE LEGENDS

Figure 1. Biochemical assays for HTS to identify MDMX inhibitors. (A) Schematic of the fluorescence polarization (FP) assay used to identify MDMX inhibitors. The protein used for the screen consisted of residues 1-185 of MdmX fused to GST. The peptide (orange) was conjugated to FITC (green) for the primary screen and to Texas Red for secondary assays. (B) Plot of the percentage of bound p53–FITC peptide (at a fixed concentration) with increasing concentrations of MDMX (squares) or MDM2 (triangles). (C) Plot of the percentage of p53–FITC peptide associated with indicated proteins in the presence of increasing concentrations of unlabeled wild-type p53 peptide or unlabeled alanine-substituted p53 peptide (AAA-p53) as a negative control. (D) Plot of the percentage of p53–FITC peptide associated with the indicated proteins in the presence of increasing concentrations of nutlin-3a. (B–D) Each data point is the mean and standard deviation of triplicate experiments. (E) Scatterplot of HTS of a chemical library for MdmX inhibitors. The blue data points indicate compounds that were selected for further analysis, and the black data points are compounds that did not exhibit activity in the HTS. The unlabeled AAA-p53 peptide (red) was used as a negative control, and the unlabeled p53 peptide (green) was used as a positive control. The density plot illustrates the clear separation of the positive- and negative-control samples across the entire screen. Each day of screening is separated by a yellow line.

Figure 2. Identification of diverse chemotypes with candidate MDMX inhibitors. (A) Work flow schematic of the primary HTS, secondary analysis, and dose-response and cell-based assays. Numbers of compounds that were selected for each round of analysis are indicated. (B) Histogram of distribution of 1,152 compounds based on percent inhibition of MDMX activity. Gray bars represent data from all compounds from the FITC-FP assay results in the original HTS. Black bars represent the compounds that were later validated with dose-response analysis. Light blue bars are data from the triplicate Texas Red FP assay, and the dark blue bars are those that were later validated with dose-response analysis. (C) Distribution of EC₅₀ values for MdmX, MDMX, and MDM2 for all 1,152 compounds calculated from the dose response in triplicate using the Texas Red FP assay. Eleven compounds were then selected for further characterization from 7 of the chemotype clusters. (D–J) The Murcko scaffolds for the 7 clusters are shown. The black triangles are compounds related to that Murcko scaffold, and each gray line from those triangles represents a compound in the plate. The shading of the circles for each compound is related to its binding constant for MDMX, and the size reflects the selective cytotoxicity for retinoblastoma cells versus BJ cells. Large, dark blue circles are those with low binding constants for MDMX and selective cytotoxicity for retinoblastoma cells.

Figure 3. SJ-172550 reversibly binds MDMX. (A) Heat map of normalized activity for the 11 compounds selected for follow-up characterization. Dark blue is more favorable for each measurement, and the compounds are listed in order of binding constant for MdmX from best (top) to worst (bottom). These data suggested that with subsequent biochemical analyses, SJ-
134433 and SJ-044557 were less suitable for follow up, and SJ-172550 was preferred. (B, C) High-performance liquid chromatography (HPLC-UV-MS) of compound SJ-172550 showed that it is stable for 24 h in FP buffer, and (D) MALDI mass spectrometry showed that it does not covalently modify the MDMX protein. The MDMX protein was incubated with SJ-172550 for 24 h in FP buffer and then dialyzed away in a large excess of dialysis buffer. The ability of the dialyzed protein to bind the p53-FITC peptide was then measured using the FP assay (red line). (E) The EC₅₀ for binding to p53 peptide after removal of SJ-172550 was indistinguishable from that of the untreated protein. A similar experiment with nutlin-3a also demonstrated that it binds reversibly to MDMX. (F) SJ-044557 and SJ-134433, which covalently modified MDMX, did not reversibly bind MDMX (red lines).

Figure 4. SJ-172550 disrupts the MDMX-p53 interaction in cells maintained in culture. (A) An isobologram shows the additive inhibition of Weri1 cell growth when the combination of SJ-172550 and nutlin-3a was used to treat cells. Mutiple ratios were tested, and the plot shows that the LC₅₀ (dashed line) was additive but not synergistic (shaded area) or adverse (area above the dashed line). The error bars represent standard deviation from 2 independent experiments. (B, C) Immunostaining of compound-treated Weri1 cells. The top panels are merged images of DIC and DAPI staining, and the lower panels show p53 levels (B) or Caspase-3 activation (C). Nutlin-3a and IR were used as positive controls, and DMSO was used as a negative control. (D-F) Quantification of the percentage of immunopositive cells shown in B and C and BrdU. (G) Real-time PCR quantification of p53 target genes p21, MDM2, and E2F1 activated by drug or IR treatments. MDMX levels were also tested. (H) Immunoblot and quantification for p21 protein levels activated by drug or IR treatment. (I,J) Histogram of the proportion of p53 or activated Caspase-3 immunopositive cells following treatment with 5 Gy IR, nutlin-3a or SJ-172550. In parallel samples, p53 was knocked down using a p53 siRNA, MDMX was knocked down using an MDMX siRNA or the samples were treated with a control (scrambled) siRNA. Each bar represents the mean and standard deviation of scoring in triplicate o 100 cells for each condition and each siRNA. (K) Representative images of Weri1 retinoblastoma cells following treatment with SJ-172550 when they lacked p53 (p53 siRNA) or MDMX (MDMX siRNA) as compared to a control siRNA. Arrows indicate activated Caspase-3 immunopositive cells in the red (Cy3) channel. Abbreviations: DIC, differential intereference contrast microscopy. Scale bars: 10 µM.

Figure 5. (A,B) A space-filling model of the overlaid MDM2-nutlin-3a (teal) with MDMX-p53 (pink) showing SJ-172550 bound to the p53-binding pocket of MDM2/MDMX. (C,D) Docking of SJ-172550 to MDMX superimposed on the crystal structure of p53 peptide bound to MDM2. The gray is the solvent excluded surface of MDM2 (PDB: 2Z5T) and the blue is the surface for MDMX. Green residues represent single mutations and residues shown in red formed a quadruple mutant. The first set of residues were changed to displace SJ-172550 based on the models of the most energetically favorable docking poses (Fig. 5D). These residues within MDMX were changed as follows: Q58D, M61F, Y66I and Q71D. A second series of residues were changed to make the MDMX binding pocket more like MDM2 in order to determine if SJ-172550 was binding in the p53 binding pocket. These residues include M53L, H54F and a quadruple mutant (QUAD) with P95H+S96R+P97K+R103Y. From top to bottom the green residues are M53, H54, Q58, M61, Y66 and Q71 with the red residues being R103, P97, S96 and P95. (E) Plot of direct binding of each MDMX protein to Texas Red labeled p53 peptide exactly as described for the high throughput screening. Each point is the mean and standard deviation of
triplicate assays. The EC$_{50}$’s for each protein are indicated. (F) Competition experiments with each MDMX mutant and increasing concentrations of SJ-172550. Each data point is the mean and standard deviation of triplicate assays. The proteins that did not show direct binding of p53 peptide could not be analyzed in this competition experiment. (G) Summary of the EC$_{50}$’s for direct binding of p53 peptide and competition by nutlin-3a and SJ-172550. The shaded column (M53L) is one of the mutants that was predicted to make MDMX more like MDM2 and increase nutlin-3a binding while inhibiting SJ-172550 binding without affecting peptide binding.
Reed et al. Fig. 1

A

B

C

D

E

Reed et al. Fig. 1

A

B

C

D

E
Reed et al. fig. 3

A

| SJ-134433 | SJ-044509 | SJ-044512 | SJ-135056 | SJ-044557 | SJ-185452 | SJ-158964 | SJ-172550 | SJ-034877 | SJ-165217 | SJ-247100 |
|------------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|
| MW: 47750  | MW: 47758 | MW: 460.14 | MW: 460.14 |

B

SJ-172550

t = 0
MW = 460.14

AU

2.53 2.89

Time

2.4 2.5 2.6 2.7 2.8 2.9 3.0 3.1 3.2 3.3 3.4

C

SJ-172550

t = 24 hr
MW = 460.14

AU

2.53 2.89

Time

2.4 2.5 2.6 2.7 2.8 2.9 3.0 3.1 3.2 3.3 3.4

D

MW: 47750
MW: 47758

m/z

46,000 46,500 47,000 47,500 48,000 48,500 49,000 49,500 50,000

E

p53 peptide
nutlin-3a
SJ-172550

EC₅₀=0.84 µM

mP

-4 -3 -2 -1 0 1 2

F

p53 peptide
nutlin-3a
SJ-044557
SJ-134433
**Reed et al. Fig. 4**

**A**

![Graph showing the relationship between nutlin-3a concentration (µM) and SJ-0172550 concentration (µM)](graph)

**B**

![Images showing different treatments: nutlin-3a, SJ-172550, untreated, 5 Gy IR](images)

**C**

![Images showing nuclei stained with DAPI](images)

**D**

![Bar charts showing p53 expression in different cell lines](charts)

**E**

![Bar charts showing caspase 3 expression in different cell lines](charts)

**F**

![Bar charts showing BrdU incorporation in different cell lines](charts)

**G**

![Bar charts showing normalized relative fold changes of p53, p21, MDM2, MDMX, and E2F1](charts)

**H**

![Images showing protein expression for p21 and actin](images)

**I**

![Images showing p53 expression with different treatments](images)

**J**

![Images showing caspase 3 expression with different treatments](images)
