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Surface plasmon resonance and cytotoxicity assays of drug efficacies predicted computationally to inhibit p53/MDM2 interaction

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Abstract

Docking on the p53-binding site of murine double minute 2 (MDM2) by small molecules restores p53's tumor-suppressor function. We previously assessed 3244 FDA-approved drugs via “computational conformer selection” for inhibiting MDM2 and p53 interaction. Here, we developed a surface plasmon resonance method to experimentally confirm the inhibitory effects of the known MDM2 inhibitor, nutlin-3a, and two drug candidates predicted by our computational method. This p53/MDM2 interaction displayed a dosage-dependent weakening when MDM2 is pre-mixed with drug candidates. The inhibition efficiency order is nutlin-3a ($IC_{50} = 97$ nM) > bepridil (206 nM) > azelastine (307 nM). Furthermore, we verified their anti-proliferation effects on SJSA-1 (wild-type p53 and overexpressed MDM2), SW480 (mutated p53), and SaOs-2 (deleted p53) cancer cell lines. The inhibitory order towards SJSA-1 cell line is nutlin-3a ($IC_{50} = 0.8$ μ M) > bepridil (23 μ M) > azelastine (25 μ M). Our experimental results are in line with the computational prediction, and the higher IC_{50} values from the cell-based assays are due to the requirement of higher drug concentrations to penetrate cell membranes. The anti-proliferation effects of bepridil and azelastine on the cell lines with mutated and deleted p53 implied some p53-independent anti-proliferation effects.

Keywords

p53; MDM2; Drug screening; Repurposing of existing drugs; Inhibitory effects

1. Introduction

As the “guardian of the genome,” p53 plays a pivotal role in regulating downstream genes that are involved in DNA repair, cell cycle arrest, and apoptosis, thereby halting the

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Appendix A. Supplementary data

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propagation of cells carrying damaged DNA [1–4]. Cellular stress (e.g. DNA damage, hypoxia, and oncogene activation) can rapidly stabilize p53 via blocking its degradation. In the absence of cellular stress, murine double minute 2 (MDM2), a 491-amino acid (AA)-long phosphoprotein, physiologically lowers p53 protein levels by binding to its transcription-activation domain [3,5]. Upon binding and through its E3-ubiquitin ligase activity, MDM2, together with MDMX, catalyzes degradation of p53 by the 26S proteasome [6]. Several mechanisms can inhibit p53 activity resulting in cancer [1], including mutation within its DNA-binding domain, binding to cancer-causing viral proteins, and abnormal MDM2 overexpression [5]. All of these disturbances result in continued proliferation of cells with damaged DNA. The direct interaction between MDM2 and p53 is enabled by attachment of p53's preformed amphipathic two β -turn domain (AA residues 15–29) [7–9] to the hydrophobic pocket of MDM2 (AA residues 25–109) [8,10,11]. More specifically, p53 forms an α -helix upon binding to MDM2, [12–14] and the three hydrophobic AA residues of p53 (Phe19, Trp23 and Leu26) are essential to its binding to MDM2 [7]. Abnormal MDM2 accumulation has been reported to block p53's degradation and induce cancers in numerous tissues [15]. One of the approaches to reactivate p53 in cancer cells that overexpress MDM2 is to dock small molecules onto the hydrophobic pocket of MDM2 and thereby rescue p53 activity [16–18].

Nutlins are compounds that share a cis-imidazoline scaffold and bind to the hydrophobic pocket of MDM2 in a fashion analogous to the combined effect of Phe19, Trp23 and Leu26 residues of p53 [19,20]. In general, nutlins antagonize MDM2 and accelerate apoptosis of cancer cells with wild-type p53 (wt-p53) [21] and, specifically, nutlin-3 antagonized MDM2 in a panel of cancer cells lines that harbor wt-p53 and reduced tumorigenicity of human breast cancer cells growing in nude mice [18] However, similar to other cancer treatment drugs, resistance to nutlin can be acquired [22].

Because it is difficult to bring novel drugs to market, [23] it may be advantageous to repurpose previously FDA-approved drugs for use against MDM2. Previously, one of us created a computational method, referred to as “computational conformer selection”, to search for possible MDM2 antagonists from a database of FDA-approved drugs [24]. A total of 3244 FDA-approved drugs (isomers and alternate protonated forms were included for the 1125 unique molecular formulas) were ranked in terms of their predicted MDM2 inhibitory efficacies [23].

Computational conformer selection affords useful pre-judgment among potential drugs, but its prediction requires experimental verification. In this work described here, we established a surface plasmon resonance (SPR) method by measuring the binding affinity between p53 and MDM2. Specifically, a synthetic p53 peptide whose sequence encompasses residues 15–29 was tagged at its N-terminus with a hexahistidine tag (His₆). The resultant peptide was immobilized onto a Ninitrilotriacetic acid (NTA) sensor. MDM2 or an MDM2/nutlin-3a mixture was subsequently flowed over the sensor and changes in the SPR binding signals were used to deduce the nutlin-3a/MDM2 binding affinity. Previously, Vassilev and co-workers attached recombinant human p53 onto a penta-histidine-specific antibody covalently affixed onto carboxymethylated dextran and used SPR to verify the effect of nutlins on inhibiting the binding of MDM2 to p53 [25]. In comparison, an attractive feature

of our method is that the sensor surface can be readily regenerated, as no antibodies were used and the His₆-tagged peptide can be completely stripped off the sensor surface using EDTA. Consequently, IC₅₀ values can be reproducibly determined and the same sensor surface can be repeatedly used for 45–49 cycles of continuous drug screening. We further extended our SPR method to the measurements of MDM2 binding affinities of two commercially available compounds, bepridil and azelastine. Bepridil was once used to treat angina and is a possible drug for treating atrial fibrillation, while azelastine is a first therapy of rhinitis [26–28]. These two drugs are among the top 15 nutlin-like drugs selected by the computational conformer selection [24]. We also assessed the anti-proliferation effects of nutlin-3a, bepridil, and azelastine with three cancer cell lines, SJSA-1 (expresses wt-p53 and high levels of MDM2), SW480 (expresses mutant p53), and SaOs-2 (p53 gene is deleted). Two of these cell lines (SJSA-1 and SW 480) were also used in the work by Vassilev and co-workers to evaluate the anti-proliferation efficacies of nutlins [25]. The SaOs-2 cell line was added to determine the effect of these drugs in the complete absence of p53. Our SPR measurements of purified MDM2 and cell-based assays both confirm that the computational conformer selection method is accurate in pre-selecting potential drug candidates for the purpose of inhibiting the p53/MDM2 interaction. Thus, we have developed in vitro and cell-based assays in conjunction with the computational conformer selection method for screening drugs that can disrupt the interaction between p53 and MDM2. Such an approach should also find applications in identifying potential drug candidates as antagonists for other types of protein/protein interactions.

2. Materials and methods

2.1. Materials

N-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), ethanolamine (EA) hydrochloride, K₂HPO₄, KH₂PO₄, NaCl, ethylenediaminetetraacetic acid (EDTA), NaOH, dimethyl sulfoxide (DMSO) and isopropyl β-D-1-thiogalactopyranoside (IPTG) were all obtained from Sigma-Aldrich (St. Louis, MO). Azelastine hydrochloride and bepridil were obtained from Sigma-Aldrich. Nutlin-3a was acquired from EMD Millipore Corp (Billerica, MA). Other reagents were of analytical purity and used as received. Solutions were prepared daily with deionized water from a Millipore system (Simplicity 185, Millipore Corp, Billerica, MA). Nitrotriacetic acid (NTA) sensor chips were purchased from Biosensing Instrument Inc. (Tempe, AZ). Azelastine, bepridil, and nutlin-3a were separately dissolved in DMSO to afford 10 mM stock solutions, which were further diluted to 10 μM with the running buffer (10 mM sodium phosphate buffer containing 150 mM NaCl and 20 μM EDTA). Each drug candidate was mixed with 400 nM MDM2 and allowed to stand for 20 min at ambient temperature before SPR experiments. The final DMSO concentration in MDM2 and MDM2/drug mixtures was 0.1% (v/v).

2.2. p53 peptide and recombinant MDM2

The N-terminal single His₆-tagged human p53 peptide with the sequence of HHHHHHSQETFSDLWKLLPEN was synthesized in house on a peptide synthesizer (Biotage Initiator, Charlotte, NC). The purification was performed on an HPLC system

equipped with a C18 column (100 × 4.6 mm, 5 μm particle size, 100 Å pore size, Phenomenex Inc., Torrance, CA). The solvent system was 0.1% trifluoroacetic acid (solvent A) and acetonitrile with 0.1% trifluoroacetic acid (solvent B), and the starting point was 7% (v/v) solvent B with a linear gradient up to 70% solvent B. The flow rate was 1 mL/min and the gradient profile was 40 min.

E. coli plasmid pGEX-2T-MDM2 (AA 3–155) expressing glutathione-S-transferase (GST)-MDM2 fusion protein was a gift from Dr. Jiandong Chen (Moffitt Cancer Center, Tampa, FL) [29]. Plasmid was transformed into *E. coli* JM109 cells and amplified. Amplified plasmid was then transformed into *E. coli* BL-21 cells and 2 L of cells were induced by 0.5 mM IPTG for 5 h at 37 °C. Cells were harvested by centrifugation at 3488×*g* and lysed in 20 mL of lysis buffer by a 30-min sonication (10-s pulse vs. 3-s rest). The glutathione agarose column (Pierce GST Spin Purification Kit, Thermo Fisher Scientific, Waltham, MA) was used to purify GST and GST-MDM2 from the soluble lysate (both GST and GST-MDM2 were expressed). The washing and elution buffers were 125 mM Tris, 150 mM NaCl, pH 8.0 and 125 mM Tris, 150 mM NaCl, and 10 mM reduced glutathione, pH 8.0, respectively. The fraction containing GST-MDM2 and GST was collected and the protein concentration was measured by Bradford assay [30]. The resultant mixture was against 50 mM Tris, 125 mM NaCl (pH 8.0) for 16 h at 4 °C. After that, thrombin was used to cut the GST tag from GST-MDM2. For 58 mg GST-MDM2/GST mixture, 1160 units of protease thrombin were used. Once complete, 1 mM phenylmethanesulfonyl fluoride was added to stop the cleavage reaction. The sample was placed in the glutathione agarose column to remove GST from the MDM2/GST mixture (Fig. S1A in the Supporting Information). The MDM2 protein sequence (AA 3–155) was confirmed by in gel trypsin digestion (Fig. S1A) and mass spectrometric analysis at City of Hope Mass Spectrometry Facility (Duarte, CA). Out of 152 MDM2 amino acid residues only 13 were not detected due to the fact that these residues reside in peptides of less than 5 amino acids in length. Size exclusion chromatography was performed using fast protein liquid chromatography (FPLC) to further purify MDM2 (Fig. S1B) with a Bio-rad enrich SEC 650 column (10 × 300 mm, operation pressure 600 psi). The running buffer was 125 mM Tris-HCl and 150 mM NaCl (pH 8.0) and the flow rate was 1 mL/min.

2.3. IC₅₀ measurement

The IC₅₀ measurements were conducted with SPR (BI-SPR 4500 system, Biosensing Instrument Inc.). Sodium phosphate buffer (10 mM) containing 150 mM NaCl, pH 7.8 was degassed under vacuum for 30 min before being used as the running buffer. The NTA sensor chips were loaded with Ni²⁺ through exposure to 5 mM NiSO₄ at 20 μL/min for 10 min. Subsequently, His₆-tagged p53 peptide was immobilized by flowing 1 μM peptide initially dissolved in running buffer at 20 μL/min over the chip for 10 min. When the baseline becomes stable after the initial desorption of excess p53 peptide 400 nM MDM2 in the absence or presence of a drug candidate was injected at 10 μL/min. The stable baseline corresponds to 264 ± 5 RU or 264 ± 5 pg/mm² of p53, a value averaged from 50 sensorgrams. Sensor regeneration was accomplished by exposing the sensor surface to 200 mM EDTA (pH = 9.0) at 60 μL/min for 100 s. IC₅₀ values were calculated from the SPR

signal/drug concentration curves using the dosage response software program of Origin 9.0 (Redwood City, CA).

2.4. Cell lines

SJSA-1 cell line (wild type p53 expressed and MDM2 amplified), kindly donated by Dr. A. Thomas Look (Dana Farber Cancer Institute, Boston, MA), was cultured in RPMI 1640 medium (Fisher Scientific, Pittsburgh, PA) at 37 °C in the humidified chamber containing 5% CO₂. The SW480 cells (p53 mutated), purchased from American Type Culture Collection (ATCC), was grown in Leibovitz's L-15 growth media (Fisher Scientific, Pittsburgh, PA) at 37 °C, in a humidified chamber with atmospheric air. The SaOs-2 cells (p53 null), kindly donated by Dr. Carlotta Glackin from City of Hope, were grown in Dulbecco's Modified Eagles Medium (DMEM) (Gibco, Invitrogen, Carlsbad, CA) in a humidified atmosphere containing 5% CO₂ at 37 °C. All media were supplemented with heat inactivated (30 min at 56 °C) 10% fetal bovine serum (FBS) (Gibco, Invitrogen, Carlsbad, CA), penicillin (100 U/I), and streptomycin (100 µg/mL) (Bio Whittaker, Lonza, Walkersville, MD).

2.5. MTT assay

The cells were grown in flasks, harvested by treatment with trypsin-EDTA solution (0.25% EDTA 1× trypsin), and counted using hemocytometer. Aliquots containing appropriate numbers of cells (previously determined by cell proliferation analysis to ensure cells were in the log-phase growth during drug treatment) were added to inner wells of a 96-well plate. Cells were allowed to grow for 36 h prior to drug treatment. Increasing concentrations of selected drugs (dissolved in DMSO/media) were added to respective wells. All dosage groups were assayed in triplicate. After five days of incubation, old media was aspirated and fresh media with MTT 5 mg/mL (to a final concentration of 0.5 mg/mL) was added to each well and incubated for an additional 3.5 h. The medium was replaced with 50 µL of DMSO and agitated for 30 min at room temperature. Absorbencies were measured on a VictorX plate reader using a test wavelength of 570 nm and a reference wavelength of 690 nm. The 690 nm signal was subtracted from that at 570 nm to correct for background noise. The 50% inhibitory concentration (IC₅₀) was extrapolated from the concentration–effect curves by linear regression analysis (MS Excel Program). To exclude the edge effects on the 96-well plate, which is known to cause increased variability in sample readings, all outer wells were filled with growth media without cells. The drug doses were optimized for each drug in the range of 0–25 µM for nutlin 3a, which served as the positive control, and in the range of 0–100 µM for bepridil and azelastin. Control cells were treated with concentrations of DMSO equivalent to the final concentrations used to dissolve drugs in solvent.

3. Result and discussion

3.1. SPR measurements of p53 and MDM2 interaction in the presence and absence of drugs

Fig. 1A and B are representative SPR sensorgrams demonstrating that azelastine inhibits MDM2 binding to p53. By introducing a histidine tag at the N-terminal of the peptide, p53 can be immobilized onto the Ni-NTA chip surface without affixing the lysine existing in the

α -helix sequence. Moreover, we utilized NTA chips based on the following considerations: (1) the His₆-tagged p53 peptide/Ni²⁺ chelation allows the MDM2 binding domain on p53 peptide to be controllably tethered to the surface for more efficient binding [31], and (2) the entire p53/MDM2 complex can be detached from the sensor chip using EDTA, thus completely regenerating the sensor surface.

The sensor surface can be quantitatively regenerated, as evidenced by the recovery of the original baseline upon EDTA injections after the elutions of the EDTA solution in Fig. 1A and B. The small change in the baseline before p53 injection and after EDTA regeneration was caused by the removal of Ni²⁺ ions. A single chip can be used for many replicate measurements (45–49 screening cycles, during which the amounts of repeated p53 immobilization decreased by less than 10%). Therefore, accurate cross comparisons among different drug candidates can be conducted. From Fig. 1B, it is apparent that the signal associated with the MDM2 binding in the presence of 500 nM azelastine (Fig. 1B) is smaller than that in the absence of azelastine (Fig. 1A). This decrease indicates that, by docking on the p53-binding site of MDM2, azelastine has inhibited the p53/MDM2 interaction. The extent of inhibition of the p53/MDM2 interaction by 500 nM azelastine is $37 \pm 9\%$, deduced from five replicate measurements. Fig. 1C is a plot of SPR equilibrium binding signals vs. different MDM2 concentrations. From this plot, the K_d value (affinity constant) for the p53/MDM2 interaction was deduced to be 86.5 ± 35.2 nM, which is within the range of the reported values, 60–700 nM [32]. The association and dissociation rate constants, k_{on} and k_{off} , are listed along with this K_d value in Table 1. Fig. 1D is an overlay of binding sensorgrams in the absence and presence of 100 nM azelastine, bepridil, and nutlin-3a. The affinity constants deduced for the p53/MDM2 interaction in the presence of these three drugs are deduced similarly to that we obtained from sensorgrams depicted in Fig. 1C. As can be seen from Table 1, the inhibitory efficiencies of the three drugs are nutlin-3a > bepridil > azelastine, on the basis of the increasing K_d values. The association and dissociation rate constants (k_{on} and k_{off}) are useful for pharmacokinetic studies. A higher k_{off} value is indicative of a more rapid dissociation of MDM2 from p53. This trend is consistent with the predication by our computational conformer selection [24].

3.2. IC₅₀ values of nutlin-3a, bepridil, and azelastine

To quantify the inhibition of the MDM2/p53 interaction by nutlin 3a, bepridil, and azelastine, we determined their IC₅₀ values (half maximal inhibitory concentrations) by premixing 400 nM MDM2 with a drug prior to injection onto immobilized His₆-tagged p53 peptide. From the dose response curves (Fig. 2), the IC₅₀ values were deduced to be 367 nM for azelastine, 206 nM for bepridil, and 97 nM for nutlin-3a. The IC₅₀ value for nutlin-3a is in excellent agreement with that reported in literature (90 nM), [25] validating the reliability of our method.

3.3. Anti-proliferation effects of nutlin-3a, bepridil and azelastine

While a compound can block the p53 and MDM2 interaction in vitro, it might not work in vivo. The next step of drug development is to test their effects on cancer cells as some drugs lack membrane-penetrating ability. To assess the anti-cell proliferation effects of the three selected drugs and to determine their IC₅₀ values at cellular levels, exponentially growing

SJSA-1 (osteosarcoma cell-line with wt-p53 genotype and MDM2 overexpression [33]) were first incubated with these drugs. A dose-dependent decrease in cell viability was observed (Fig. 3A). The IC₅₀ values determined for nutlin-3a, bepridil, and azelastine are 0.8, 23, and 25 μ M, respectively. In addition, the DMSO vehicle does not show cytotoxic effects at concentrations less than 50 μ M, which is significantly higher than the IC₅₀ values of the drugs. Note that the IC₅₀ values calculated from the cell-based assays are about one order of magnitude greater than those measured using SPR. This could be attributed to a requirement for higher concentrations needed to penetrate the cell membrane or perhaps that there are other drug targets within the media/serum or in the cells. Nevertheless, the order of inhibition determined from the cell-based assay is consistent with the SPR measurements. We also attempted to inhibit cell proliferation with protirelin, another drug identified by computational conformer selection as a strong MDM2 binder [19]. However, we found no evidence for decreased cell proliferation (data not shown), indicating that this drug may be blocked from reaching MDM2 in cultured cells or may not have the correct properties to properly engage with MDM2.

We observed that the SJSA-1 cells treated with bepridil or azelastine did not result in a sigmoidal dose response curve, but rather a polynomial shaped curve (Fig. 3A). This dose-response curve indicates that the drugs at relatively low concentrations appeared to show initial anti-proliferation effects and at high concentrations exert cytotoxicity. Such a dose-response curve is characteristic of a hormetic response where compounds at low doses will exert a response opposite of that at which a high dose is administered [34,35]. This observation prompted us to carry out a more detailed evaluation of the drug response across 0.29–4.7 μ M, which is the range of concentrations that cause the initial anti-proliferation response. The data are shown in Fig. 3B, which further confirmed the occurrence of the hormetic response. SJSA-1 cells are subject to an initial cytotoxicity to 1 μ M bepridil and 1.5 μ M azelastine, with cell proliferation inhibited by 20% and 30%, respectively. At medium concentrations (above 3 μ M) 90% and 98% of cells were viable in the presence of bepridil and azelastine, respectively. At 12.5 μ M 30% growth inhibition was observed for both drugs, indicating that the drugs inhibited cell growth with high efficacy.

Hormetic dose responses are well known and commonly observed in human tumor cell lines where low and high doses of drug have opposite effects and activate different signaling pathways [36]. Hormetic apoptotic responses have been reported in irradiated zebrafish embryos and in ouabain/veratridine-treated Neuro-2A cells in response to brevetoxin [37,38]. The brevetoxins are thought to act through the activation of voltage-sensitive sodium channels in cell membranes [38].

We also examined the anti-proliferation effects of the three drugs on SW480 cells, a colon adenocarcinoma cell line that expresses mutant p53 and is expected not to respond to drugs. The candidate drugs considerably decreased the SW480 cell viability. The IC₅₀ values determined for nutlin-3a, bepridil, and azelastine are 0.3, 5, and 11 μ M, respectively. As presented in Fig. 3C, nutlin-3a shows an immediate decrease in cell viability up to 65% even at concentration as low as 0.024 μ M. However, after the initial decrease in cell viability (reaching 75% inhibition), the cell viability does not show appreciable changes as the drug concentration increases. In fact, at the maximum concentration of 25 μ M the viability is

about 31%, suggesting that nutlin-3a has become ineffective. Bepridil and azelastine caused significant decreases in cell proliferation at the concentration of 25 μ M (over 90% of cells were not viable).

It is intriguing that nutlin-3a decreases SW480 viability because MDM2 levels are thought to be low due to lack of wild-type p53, the major transcriptional activator of MDM2 gene. Some reports suggest that although SW480 is a *Tp53* double mutant cell line (one of its mutations lies within important DNA binding domain), it can retain proficiency for some p53 functions [39,40]. It has been reported that the mutated p53 tetramer in SW480 cells was able to bind consensus DNA sequence in vitro, remove cyclobutane pyrimidine dimers induced by UV light via nucleotide excision repair (NER), and repair damaged DNA [39]. Furthermore, the fact that our results show that the MDM2-specific drug nutlin-3a significantly decreased cell viability (up to 75% inhibition) strongly suggests that MDM2 is active and supports the observations that bepridil and azelastine are also capable of inhibiting cell proliferation by binding to MDM2. Nevertheless, the unexpected results prompted us to test the cytotoxic effects of selected drugs on the third cell line, the SaOs-2, in which p53 is deleted.

As shown in Fig. 3D, treatment of SaOs-2 cells with nutlin-3a decreases cell viability with an IC_{50} of 25 μ M. Interestingly, bepridil and azelastine are inhibitors of SaOs-2 cell growth with an IC_{50} values of 10 μ M and 15 μ M, respectively. The data suggest that all three drugs have off-target cell growth effects at 25 μ M and higher in a p53-independent manner.

Table 2 summarizes the IC_{50} values. In SJSA-1 cells nutlin-3a is approximately 29-fold more effective than bepridil and 30-fold more effective than azelastine in inhibiting cell proliferation. The order of inhibitory effectiveness reflects the order of potency in inhibiting MDM2 binding to p53: nutlin-3a > bepridil > azelastine, as these cells express MDM2 and wild-type p53. SW480 cells express only mutant forms of p53. However, it has been reported that some wild-type p53 function is retained in these mutants. In SW480 cells, nutlin-3a is 17-fold more effective than bepridil and 37-fold more effective than azelastine in inhibiting cell proliferation, again reflecting the order of potency of inhibition of MDM2 binding to p53. Finally, in SaOs-2 cells bepridil is more effective than nutlin 3a and azelastine in inhibiting cell proliferation. Nutlin-3a is the least effective with an IC_{50} of 25 μ M, reflecting an off-target of cell growth inhibition. In Table 2 we also listed the Tanimoto and docking scores, along with the Pearson's r values to reflect on the correlations between the IC_{50} values of the three drugs and the two types of scores. Tanimoto score is a gauge of the similarity of the molecules and the docking score is used to estimate the binding energy associated with the docking of a drug to MDM2. As nutlin-3a is a model system in our previous work, we set its Tanimoto score as 1 and used this value to calculate Pearson's r values. It is apparent that IC_{50} values correlate better with the Tanimoto score than with the docking score. Our data indicate that, while bepridil and azelastine are 2-fold and 3-fold, respectively, less effective than nutlin-3a in preventing binding of MDM2 to p53 in vitro, they are more than a magnitude less effective in inhibiting cell proliferation. It is likely that nutlin-3a is fairly specific in targeting p53 and that bepridil and azelastine have off-target effects that contribute to their antiproliferative activities.

4. Conclusions

Using surface plasmon resonance, we have successfully confirmed the order of efficiency in inhibiting the p53/MDM2 interaction predicted by the computational conformer selection for three select drugs, nutlin-3a, bepridil and azelastine. An attractive feature of our SPR experiment is the method of immobilization of histidine-tagged p53 peptide onto the Ni-NTA sensor. As the chelated p53 and MDM2 molecules on Ni-NTA surface can be completely regenerated, the interaction between the recombinant MDM2 and p53 peptide in the presence of different dosages of a given drug can be repeatedly measured, allowing the inhibition efficiencies (IC₅₀ values) to be reproducibly and accurately measured. For the cell viability assays, we found that these three drugs possess anti-proliferation effects on cell lines expressing mutated p53 (SW480), no p53 (SaOs-2), and wild-type p53 and high levels of MDM2 (SJSA-1). That bepridil and azelastine affect the viabilities of the SW480 and SaSO-2 cells suggests that at least some of the anti-proliferation effects are p53 independent. Although bepridil and azelastine have different IC₅₀ values for inhibiting the p53 and MDM2 interaction in vitro, their anti-proliferation effects in vivo are comparable. This reflects the need to consider drug permeability across cell membrane and the effect of cellular milieu on the interaction between drugs and MDM2 (as well as that between p53 and MDM2) for drug screenings. In summary, our experimental results demonstrate that the computational conformer selection method, when used in conjunction with in vitro measurements of purified protein and cell viability assays, can be a cost- and time-effective approach as the first step towards repurposing FDA-approved drugs for treating other types of diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Fig. 1.

SPR sensorgrams corresponding to the immobilization of 1 μM His₆-tagged p33 peptide (injections denoted by the blue arrows in panels A and B) and the injection of 400 nM MDM2 without (A) and with (B) mixing with 500 nM azelastine (red arrows). EDTA (200 mM, pH = 9.0) was injected (black arrows) to regenerate the sensor chip. Note that the injections of NiSO₄ were indicated with a green arrow and the large EDTA-induced response peaks are truncated. (C) A plot of SPR signals corresponding to binding equilibria between pre-immobilized p33 and MDM2 against the five different MDM2 concentrations (100, 200, 300, 400 and 500 nM). (D) Overlaid sensorgrams showing the binding of MDM2 alone (green), and MDM2 mixed with 100 nM azelastine (blue), bepridil (red), and nutlin-3a (black) prior to injection onto chips immobilized with the His₆-tagged p33 peptide. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Fig. 2.

The percent inhibition of MDM-2 binding to His₆-tagged p53 peptide by nutlin-3a (A), bepridil (B), and azelastine (C) measured by SPR (empty circles), along with the fitted dose response curves (red). The concentrations used were 10, 20, 50, 100, 500, and 1000 nM for nutlin-3a; 10, 50, 100, 200, 500, and 1200 nM for bepridil; and 100, 300, 400, 600, 800, and 2000 nM for azelastine. Each concentration was repeated at least three times and the error bars correspond to the relative standard deviations. The R^2 values for the dose response simulations are also shown. The corresponding structure of each drug is shown on top of the respective inhibition curve. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Fig. 3.

Anti-proliferation effects of nutlin-3a, bepridil, azelastine, and DMSO (vehicle control) on SJSA-1 (A), SW 480 (C), and SaOs 2 (D) cells. Panel (B) is an enlarged view of the blue and red curves in panel (A). Each point is an average of three replicates and the error bars correspond to the standard deviations. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Kinetic and affinity constants of the p53-MDM2 interaction in the presence of three drugs.

	MDM2	MDM2/100 nM nutlin-3a	MDM2/100 nM bepridil	MDM2/100 nM azelastine
k_{on} ($\times 10^{-3} \text{ M}^{-1} \text{ s}^{-1}$)	3.5 ± 0.1	4.4 ± 0.6	3.6 ± 0.7	2.4 ± 0.4
k_{off} ($\times 10^4 \text{ s}^{-1}$)	2.5 ± 0.5	8.9 ± 1.5	6.7 ± 1.0	2.6 ± 0.3
K_d (nM)	86.5 ± 35.2	208.0 ± 50.1	175.1 ± 30.5	108.5 ± 25.8

Table 2

Pearson's correlation coefficients between IC₅₀ values and computational scores.

	Tanimoto score	Docking score	IC ₅₀ in vitro (μM)	IC ₅₀ SJSA-1 (μM)	IC ₅₀ SW480 (μM)	IC ₅₀ SaOs-2 (μM)
Nutlin-3a	1.000	-7.7	0.097	0.8	0.3	25
Bepridil	0.748 ± 0.057	-6.66	0.206	23	5	10
Azelastine	0.726 ± 0.064	-7.28	0.367	25	11	15
Pearson's r vs. Tanimoto score			-0.846	-1	-0.867	0.919
Pearson's r vs. Docking score			0.298	0.759	0.336	-0.955