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Identification of novel small molecule inhibitors of the XPA protein using *in silico* based screening

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Abstract

The nucleotide excision repair pathway catalyzes the removal of bulky adduct damage from DNA and requires the activity of more than 30 individual proteins and complexes. A diverse array of damage can be recognized and removed by the NER pathway including UV-induced adducts and intrastrand adducts induced by the chemotherapeutic compound cisplatin. The recognition of DNA damage is complex and involves a series of proteins including the xeroderma pigmentosum group A and C proteins and the UV-damage DNA binding protein. The xeroderma pigmentosum group A protein is unique in the sense that it is required for both transcription coupled and global genomic nucleotide excision repair. In addition, xeroderma pigmentosum group A protein is required for the removal of all types of DNA lesions repaired by nucleotide excision repair. Considering its importance in the damage recognition process, the minimal information available on the mechanism of DNA binding and the potential that inhibition of xeroderma pigmentosum group A protein could enhance the therapeutic efficacy of platinum based anticancer drugs, we sought to identify and characterize small molecule inhibitors of the DNA binding activity of the xeroderma pigmentosum group A protein. *In silico* screening of a virtual small molecule library resulted in the identification of a class of molecules confirmed to inhibit the xeroderma pigmentosum group A protein-DNA interaction. Biochemical analysis of inhibition with varying DNA substrates revealed a common mechanism of xeroderma pigmentosum group A protein DNA binding to single-stranded DNA and cisplatin-damaged DNA.

Introduction

Xeroderma pigmentosum group A (XPA) is a 31 kDa protein that is required for the nucleotide excision repair pathway (NER), the main pathway mammalian cells use for the repair of bulky DNA adducts (1). Inactivating mutations in XPA result in a NER null phenotype and, in humans, the disease xeroderma pigmentosum (XP) (2). XPA is a component of the pre-incision complex involved in the recognition of damaged DNA and has been shown to contain domains that interact with several other proteins in the pathway, including replication protein A (RPA), ERCC1, and XPC-Rad23B (3). Once initial damage

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recognition has occurred, the coordination of several proteins is required for incision and removal of damaged DNA including TFIIH and the XPG and XPF/ERCC1 nucleases. Following excision of the damaged strand, the 3'OH resulting from XPF/ERCC1 incision is extended by DNA polymerase δ or ϵ followed by ligation by DNA ligase I. In addition to ligation by DNA ligase I, an alternative ligation pathway has been demonstrated which employs XRCC1 and DNA ligase III (4).

XPA's role in damage recognition has been studied extensively and it has been shown to interact with both damaged and undamaged DNA (5;6). DNA binding activity has been shown to reside in a 122 amino acid minimal DNA binding domain (MBD) spanning from M98 to F219 that contains a class IV, C4-type zinc-binding motif (7–9). A separate study shows that this cleft overlaps with the region for RPA p70 binding as well, supporting the possible cooperative model of DNA-binding between XPA and RPA (10). The overall structure of the zinc-binding domain varies from those of other zinc finger domains, however, the local four cysteine residues contained in this domain are similar to the zinc-fingers found in the GATA-1 transcription factor (7). XPA's essential role in NER is a function of DNA interactions and potentially interactions with other NER proteins.

Clinical XP is characterized by an increased predisposition to cancer and extreme sensitivity to UV-light (11). There are 7-complementation groups A-G with XPA being the most severe and having the greatest sensitivity to UV-light and other DNA damaging agents including cisplatin. Consistent with this fundamental role in NER catalyzed repair, increased XPA expression has been associated with decreased sensitivity to DNA damaging chemotherapeutic agents (12). Specifically, increased sensitivity to cisplatin therapy in testicular cancer cells has been linked to decreased levels of XPA, which results in decreased levels of NER activity and overexpression of XPA in these cells results in a more resistant phenotype (12).

Cisplatin is a common chemotherapeutic used in the treatment of several cancers including lung, ovarian and testicular cancers (13). Lung and ovarian cancer patients represent one of the highest mortality rates of all cancer patients diagnosed every year. Currently, cisplatin is a component of the first-line treatment for patients diagnosed with advanced stage non-small cell lung cancer (NSCLC); however, response rates vary and are often short-lived (14). However, no other treatments have been shown to be more effective and thus a large majority of these patients will receive cisplatin in the course of their therapy (15). Although cisplatin is a front line therapy in the treatment of NSCLC, efficacy varies significantly between patients causing a spectrum of responses. Differences in the metabolism and uptake of cisplatin as well as the repair of cisplatin-DNA lesions represent a few of the factors thought to influence cisplatin sensitivity (16;17). While a direct correlation of clinical resistance with differential expression of individual NER proteins has not been established, the decreased expression of ERCC1 has been correlated with a better prognosis and response to cisplatin based therapy following surgery (18). Overall these data suggest that by decreasing NER capacity, one could increase sensitivity to cisplatin and potentially approach clinical efficacy observed in testicular cancer response to cisplatin where 95% of patients are cured by a cisplatin based regimen (13).

Targeting protein-DNA interactions has only recently been reported in a small number of studies. Peptide based molecules were used recently to target the sequence specific Notch transcription factor (19). Small molecules have also been identified via a high throughput screen (HTS) using fluorescence polarization (FP) as a readout targeting the sequence specific DNA-binding transcription factor HOXA13 (20). In addition, we have reported the development of fluorescent based HTSs to identify SMIs of the damage-specific DNA binding proteins, RPA and XPA (21–23). In this report we present an *in silico* screen for

small molecules that target the DNA binding domain of XPA. A 3-D structure obtained via NMR of the XPA minimal DNA binding domain (MBD) reveals a cleft that includes a number of conserved basic amino acids which, using chemical shift perturbation experiments, has been suggested to be in direct contact with the DNA in conjunction with surrounding residues (24). In addition, Lys 141 and 179, found within this cleft, were shown to impact binding to kinked DNA substrates, presumably similar to those formed by bulky DNA adducts that are repaired by NER (25). The characteristics of this cleft make it an ideal site for small molecule drug screening with the goal of identifying small molecules that bind to this cleft and disrupt the direct interaction between XPA and DNA and potentially cooperative DNA binding effects between RPA and XPA as this region also interacts with RPA (10). In this study, we performed a structure-based *in silico* screen targeting this cleft and identified small molecules capable of inhibiting the XPA-DNA interaction. Characterization of these molecules provides proof-of-concept for targeting a DNA binding activity via *in silico* analysis and reveals similar mechanisms of XPA binding to structurally diverse DNA substrates.

RESULTS AND DISCUSSION

Targeting nucleotide excision repair

The NER pathway is responsible for the removal of a vast array of DNA damage from the human genome including environmental DNA damage such as that caused by exposure to UV-light and chemotherapy induced DNA damage typified by the agent cisplatin (4;26). This pathway requires the concerted activity of over 30 proteins and despite *in vitro* reconstitution of the pathway over 15 years ago, the fundamental mechanism required for many of the steps remains elusive (3;27). This is no more apparent than in the initial step of DNA damage recognition. The damage recognition step in NER is complicated by the requirement to respond to a wide array of DNA damage as such that unforeseen chemical insults to DNA can be repaired. Thus recognition must rely not on a single chemical modification but on the gross distortion in the DNA structure induced by DNA damage. This issue together with the bifurcation of recognition into two pathways, transcription coupled NER (TC-NER) and global genomic NER (GG-NER) results in a further increase in complexity (Figure 1). The role of the XPA protein in DNA damage recognition is well established though the mechanistic and temporal regulation of how this protein participates in the process remains less well defined. Considering the importance of XPA in the recognition of cisplatin DNA damage and the demonstration that reducing XPA can sensitize cells to this therapeutic agent raises the possibility that small molecule inhibitors (SMIs) targeting XPA may have therapeutic potential to increase the efficacy of cisplatin based therapies and reverse resistance that results from efficient repair of cisplatin-induced DNA lesions.

In silico screening to identify small molecules which interact with XPA's DNA binding domain

Previous high throughput assay-based screening of a small molecule library identified a number of compounds with putative inhibitory activity towards the XPA-DNA interaction (23). However, verification of this inhibitory activity in secondary screening assays was largely unsuccessful (data not shown). We therefore undertook an *in silico* screening approach towards the discovery of novel compounds that can interact with XPA and hold the potential to inhibit the DNA binding function of XPA. Using the 3D structure of XPA determined by NMR (1XPA) the coordinates of the C-terminal subdomain (residues 131 to 210) that have been shown to be in direct contact with a DNA ligand were employed in the *in silico* analysis. The cleft consisting of residues 138–142, 165–171, 174 and 177–181 was chosen as the targeted area for small molecule docking (Figure 2). The ChemDiv virtual

library was screened as described in Methods and Materials and 63 putative inhibitors of XPA's DNA binding activity were identified and subjected to a secondary *in vitro* activity analysis to directly test their ability to interact with XPA and inhibit the XPA-DNA interaction. FP analysis using purified XPA (28) and a fluorescein labeled single strand DNA substrate was performed. Of the 63 compounds identified in the *in silico* screen, 3 were found to inhibit XPA's DNA binding activity *in vitro* (Figure 3A) (23;29). Each of the compounds with verified *in vitro* activity contains a benzoic acid substituent and in the case of X80, two such moieties at either end of the molecule (Figure 3B). Compound X57 contains a methoxy-benzene substituent with a sulfomyl separating the benzoic acid (Figure 3C). Compound X60 contains a nitrobenzene distal to the benzoic acid bridged by an unsaturated amine spacer (Figure 3D). Interestingly, compounds identified in the original HTS did not share any structural characteristics with the SMIs identified by the *in silico* screen and confirmed by *in vitro* analysis. For example, SMIs X80, 57, and 60 all contain at least one acidic moiety whereas compounds identified by the HTS in general lacked this functional group. This data demonstrate the utility of *in silico* screening to identify SMI of XPA.

***In vitro* characterization of putative XPA inhibitors: fluorescence polarization and DNA substrate specificity**

In silico analysis identified compounds which putatively interact with XPA's DNA binding domain, however, preliminary *in vitro* fluorescence polarization (FP) analyses were used to confirm the interaction of each compound with XPA and inhibition of DNA binding activity. We therefore extended these FP studies to assess potency and specificity for different DNA substrates to which XPA has been shown to bind. We employed single-strand DNA and fully duplex DNA that were either undamaged or contained a single site specific cisplatin 1,2 d(GpG) adduct centrally located in the duplex. Each of these substrates was prepared with a 5'-fluorescein label and XPA was titrated with each DNA substrate to determine the concentration required to bind 70% of the input DNA. Each compound was then titrated from 0–100 μ M with a fixed concentration of XPA to determine if the compound inhibited XPA's DNA binding activity. The data presented in Figure 4 measuring binding to the single stranded DNA substrate reveal that X80 displays the greatest inhibitory activity while both X57 and X60 show less inhibition (Panel A). The same trend is also evident in the analysis of XPA binding to duplex undamaged DNA and a duplex DNA containing a single 1,2 d(GpG) cisplatin-DNA adduct (Panels B and C, respectively). The data were quantified for compound X80 on the three different substrates and IC₅₀ values calculated using a 3-parameter hyperbolic decay with offset (Table 1). There was no statistical differences observed in the calculated IC₅₀ values which suggests a common mode of XPA binding to each DNA substrate that was equally inhibited by X80. IC₅₀ values for compounds X57 and X60 were not calculated because the compounds never resulted in inhibition of XPA's DNA binding ability by more than 50%, indicating a weaker interaction with XPA compared to X80. While FP is a highly accurate and quantitative assay for DNA binding, the potential for interference as a result of spectroscopic properties of the compounds led us to assess the effect of these three inhibitors in a non-spectroscopic assay.

***In vitro* inhibition of XPA's DNA binding activity as determined by modified ELISA assay**

In order to confirm the *in vitro* activity of the three lead small molecule inhibitors, an ELISA assay was used to assess the ability of these compounds to prevent XPA from binding DNA. In this assay, biotin-conjugated 60 base single-strand DNA was bound to streptavidin coated ELISA plates. Purified XPA was titrated into the binding reactions to determine the concentration that resulted in approximately 50% XPA DNA binding activity, which was determined to be 10 ng of protein (data not shown). XPA was then pre-incubated with either vehicle or compound and then added to the wells containing the biotin conjugated DNA.

Following incubation, the plates were washed and bound XPA detected using an anti-XPA polyclonal antibody followed by an HRP conjugated goat anti-rabbit IgG secondary antibody. HRP activity was determined kinetically using a TMB based substrate as described in the Methods section. Initial analyses at relatively high concentrations revealed that at concentrations up to 200 μ M, no inhibition of binding was observed with compounds X57 and X60 (Figure 5A) while X80 inhibited XPA binding by 95% at a concentration of 100 μ M. The ELISA assay requires a greater affinity to detect an interaction than the FP assay. The complex must be stable during the washing and probing reactions and thus it is not a true equilibrium binding assay. However, the analyses do give an accurate measure of the portion of XPA that is capable of binding the immobilized substrate. That X57 and X60 do not display inhibitory activity in this assay is consistent with low inhibitory activity observed in the FP assay. Confirmatory analysis of the inhibitory activities of these compounds was obtained using a photocrosslinking approach as we described previously in the analysis of XPC-Rad23B binding to cisplatin damaged DNA (30). Binding of XPA to a duplex DNA substrate containing a single cisplatin 1–2 d(GpG) adduct modified with a single photoactivateable dCTP 5' of the lesion was assessed. Increasing concentrations of each inhibitor was included and the DNA-protein complexes crosslinked and products resolved by SDS –PAGE and visualized by phosphorimager analysis. The data confirm inhibitory activity of X80 with only less inhibition observed for X57 and X60 (Supplemental Figure S1).

Based on this analysis, X57 and X60 were not analyzed further for *in vitro* activity and X80 was assessed on each of the substrates, single stranded DNA, duplex undamaged DNA and a 1,2 (GpG) cisplatin-damaged duplex DNA (Figure 5B). These data demonstrate that X80 again displayed similar inhibitory activity when comparing inhibition of XPA on the single strand, duplex damaged DNA and undamaged duplex DNA substrates (Figure 5C,D). The calculated IC_{50} values (Table 1) did not reveal a significant difference among the DNA substrates. This result is again consistent with the FP data and indicates that the compound is interacting with XPA in a way that prevents its binding to various types of DNA substrates. The close concordance between the two independent assays suggests that the interaction of X80 with XPA disrupts the ability of XPA to bind to each of the DNA substrates tested and that the mechanism of XPA binding to each DNA substrate is similar.

Considering the relatively high concentrations necessary to inhibit XPA, the potential for aggregation of the compound inducing non-specific inhibition can be a concern. High speed centrifugation of the compound however did not result in precipitation of the compound (data not shown) thus ruling out the possibility that gross aggregation or the presence of a precipitate is responsible for inhibition of XPA. While insoluble aggregation was ruled out, soluble aggregation has also been reported for some chemical compounds identified from *in vitro* HTS (31–33). Importantly, both the FP and ELISA based DNA binding assays are performed in a buffer that contains a low level of non-ionic detergent (0.01% NP40) to disrupt potential aggregation of the small molecules. The use of these conditions ensures that any inhibition observed was not the result of soluble aggregation (34). In addition, analysis of the shape of the inhibition curves is not consistent with aggregation of the compounds which would be expected to yield a considerable steeper hill slope of inhibition over a smaller range concentration range (33).

Interrogation of SMI interactions with XPA

To assess the validity and accuracy of the *in silico* screening and docking analysis, additional compounds in the X80 class were obtained from the ChemDiv Library and assessed for XPA inhibition. The derivatives contained modified moieties in place of the substituent on each phenyl ring of X80, providing insight into the importance of the acidic groups. Compound X801 replaces the furanyl 2-chlorobenzoic acid with a 3-chlorophenyl

while compound X802 replaces the pyrazole 3-carboxyphenyl in parent X80 compound with a 3-bromophenyl. Both of these modifications resulted in a significant decrease in XPA inhibitory activity with IC_{50} values greater than 0.1 mM as assessed in the ELISA assay (Table 2). That X80 is more active than these two derivatives provides support for the accuracy of the *in silico* screen as the derivatives, X801 and 802 were ranked lower based on binding characteristics and ΔG values compared to X80. These data also demonstrate that each of the acidic functional groups is critical to XPA inhibitory function. The third derivative investigated replaced the furanyl 2-chlorobenzoic acid with a 2-hydroxy-5-nitrophenyl. This compound exhibited intermediate inhibitory activity with an IC_{50} of approximately 75 μM , consistent with the importance of a negatively charged or acidic functional group (Table 2) and again providing further support for the *in silico* screen and selection process. Importantly, minor changes in substitutions on the furan benzene including removal of the *para*-Cl or replacing the carboxyl with an ester moiety resulted in very similar inhibitory activity to the parental X80 compound.

To further investigate the impact of chemical structure on XPA inhibitory activity, we first analyzed component molecules in the X80 structure. Both the 3-(3-methyl-5-oxo-4,5-dihydro-1H-pyrazol-1-yl)benzoic acid and 2-chloro-5-(5-formylfuran-2-yl)benzoic acid displayed no inhibitory activity as expected (data not shown). Considering the relative importance of the benzoic acids moieties, we identified small molecules with similar terminal functional groups tethered by a different core structure. Compounds X055 and X126 each contain a modified phenyl bridged by a thiazolidin core structure. The data presented in Figure 6 demonstrate that X055, which contains a 2-bromo1methoxybenzene moiety, displays significantly greater activity against XPA compared to the X126 which contains a 2-(2-chloro-6-methoxyphenoxy)acetic acid substituent. That the X055 displays a greater affinity for XPA ($IC_{50} < 20 \mu M$) than X80 yet has only a single benzoic acid suggests modification to reduce overall negative charge may be possible to allow cellular uptake and activity *in vivo*.

Specificity and interactions of XPA inhibitors

To assess specificity, X80 was assessed for inhibitory activity against RPA, a protein with both single-stranded DNA binding and duplex damaged DNA binding activity. No inhibition was observed at 50 μM as assessed in an EMSA based assay (Supplemental Figure 2). Interestingly, analysis of the effect of X80 on the activity of another DNA binding protein, Ku, did reveal inhibition at 50 μM (Supplemental Figure S2). While inhibition of binding was observed, a more extensive analysis of a series of small molecules containing acidic functional groups with varying core structures revealed inhibition of Ku-DNA binding activity. This result is potentially a function of Ku's unique mode of binding DNA which involves entry from a terminus and sliding along the duplex DNA. The channel in which DNA threads is populated with a large number of basic residues that interact with the sugar-phosphate backbone of DNA and the potential ionic interactions with benzoic acid groups may impede entry of the DNA into this cavity (35).

An alternative explanation for the inhibitory data is that the X80 compound is interacting with DNA making it unable to bind to the proteins. While the chemical structure, with the presence of two negatively charged carboxyl groups, would suggest this is unlikely, we analyzed this interaction directly using a fluorescent intercalator dye displacement assay (36). The results demonstrate that the compound only alters the fluorescence at concentrations greater than 100 μM , which is attributed to the spectral properties of the compound quenching the signal (Data not shown). This demonstrates that the X80 compound is not imparting its inhibitory activity via interacting with the DNA, therefore the reduction of an XPA-DNA complex in the presence of X80 is due to a direct X80-XPA interaction.

Molecular modeling the putative interactions within the XPA MBD

As there are two known XPA structures with different conformations in loop 2, which has been shown to be directly involved in ligand binding, we next examined how X80 would interact with the two different structures by docking analysis (Figure 7A, B). The best X80 conformation docked into 1D4U (Panel B) XPA structure had a similar orientation and conformation to that in 1XPA structure (Panel A). The grid scores of X80 in the two structures are also similar with -47 and -43 kcal/mol, respectively (Table 3). We also analyzed X57 and X60 by docking into both XPA structures. As shown in Table 3, both X57 and X60 similarly did not score well in either structure. These data are completely consistent with the *in vitro* DNA binding data showing minimal inhibitory activity of X57 and X60. In addition, the difference in loop 2 conformations of the two structures does not significantly affect the calculated binding affinity of these ligands.

To gain further insight into the potential interactions between XPA and the SMIs, the data obtained from the *in silico* docking was analyzed. Inspection of the position of X57 and X60 reveal potential salt bridges between the benzoic acid carboxyl of each compound and Lys 141 and 179 (Figure 7C). Interestingly, docking of X80, the most active inhibitor, positioned the carboxyl outside the range of electrostatic interaction, but did reveal the potential for hydrogen bonds with Thr142 or Ser137. Of the three compounds, X80 was extended further through the cleft and positioned the furanyl benzoic acid carboxyl group in range for interactions with Lys137 (Figure 7D). Confirmation of the importance of this interaction was obtained by inspection of the docking results with compounds X801, 802 and 803. In X802, the carboxyl functional group of the pyrole benzene was replaced with a halogen. Docking results revealed a very similar overall position of the furanyl and pyrole ring systems and the remaining benzoic acid carboxyl group retained the ionic interaction with Lys137. Surprisingly, docking analysis of compounds 801 and 803, in which the furan benzoic acid is replaced with either a halogen or nitro substituted benzene, results in re-orientation of the compounds within the XPA DNA binding domain to retain the Lys137 interaction with the remaining benzoic acid carboxyl (Figure 7E and F). These data support the conclusion that each benzoic acid in the parent X80 compound is stabilized via interactions with basic amino acids and Lys137 salt bridge is a critical determinant of inhibitory activity. Interestingly, analysis of XPA structure and function demonstrated that individual mutations of Lys141 or 179 to alanine or glutamine had minimal influence on NER activity and XPA-DNA binding; however a double K141E/K179E mutation displayed reduced repair and XPA-DNA binding to linear duplex DNA substrates (25). That such a dramatic alteration in ionic character is required for abrogation of activity suggests there are overlapping redundant binding sites within the XPA DNA binding domain. This is further supported by demonstration that the double mutant is fully capable of binding 4-way junction, severely kinked DNA (25). Our chemical inhibition data further suggest a larger binding domain with additional points of contact that extends further through the cleft to encompass Lys 137. An alternative and somewhat less likely possibility is that positioning of X80 through the cleft contacting Lys137 induces a conformational change that abrogates DNA binding at a separate site. In light of these analyses it would be of considerable interest to determine if Lys137 is required for DNA binding and/or to potentiate the inhibitory activity of X80. It is important to note that these docking studies represent models for potential interactions between XPA MBD and the SMIs. Our *in vitro* analyses were all performed with full length XPA and we cannot rule out that the inhibitors interact with other sites in XPA that potentially influence DNA binding activity. Definitive proof of the site of X80 binding and specific interactions will require high resolution structural analyses.

We would anticipate that the ability to inhibit XPA *in vivo* would be of significant utility from a variety of perspectives. Importantly, *in vivo* inhibition of XPA would be expected to result in decreased repair of bulky adduct DNA damage via NER. This has potential as a

therapeutic strategy in the treatment of cancer in conjunction with DNA damaging chemotherapeutics including cisplatin which are repaired via NER. As alterations in DNA repair have been suggested to play an important and potentially targetable pathway (37), the identification of SMIs capable of disrupting this process could be extremely useful. This is currently being observed in the clinical treatment of cancer with inhibitors of the poly-ADP ribose polymerase (38). While the exact mechanisms involved in the therapeutic activity remain to be determined, the clinical activity can be dramatic and thus identifying new targets and agents to build upon these successes holds great promise.

METHODS

Structure-Based *In silico* Screening

In order to identify small molecules that can disrupt the XPA - DNA interaction, we first examined the solution NMR structures of the central domain of XPA (PDB ID code 1XPA (39) and 1D4U (40)). The loop 2 (residues 166–179), which has been shown to be directly involved in DNA binding, appears to have very different conformations in these two structures with a RMSD of 6.1 Å for CA atoms. Comparison of these two structures showed that the putative DNA binding groove in the 1XPA structure is narrower and deeper with a more closed conformation. The difference between these two structures may explain the flexibility of XPA in recognizing a wide variety of DNA lesions. The more closed and deep groove of 1XPA makes it more suitable for performing docking experiment. Consequently, the central domain of the 1XPA structure was used for *in silico* screening.

Molecular surface of the central domain of XPA was calculated by DMS program. Partial charges and protons were added to the protein by UCSF Chimera Dock Prep module (41). The small molecules used in the virtual screening are from the ChemDiv library, in which the 3D models were converted from the 2D chemical structures provided by ChemDiv catalogs using SYBYL6. More than 200,000 compounds were docked and screened to the cleft by UCSF DOCK 6 program (42). Compounds were first scored with DOCK GRID scoring function (43), following which the top 2,000 compounds were rescored by AMBER score function incorporated in DOCK 6 (44). The top 1,000 compounds were then clustered by MOE and the docked compounds were visually examined using the UCSF Chimera ViewDock function. Approximately 100 compounds were identified based on GRID and AMBER scores, drug likeness (Lipinski's Rule of Five), visual examination of the docked protein-compound complexes and with consideration of maximizing compounds from different clusters.

Chemicals

Compounds identified were purchased from ChemDiv or SPEX and prepared at 10 mM in 100 % DMSO. Confirmation of the structure and purity of TDRL-X80 was determined by LCMS analysis.

Overexpression and purification of human [His]₆-XPA

[His]₆-XPA was purified as previously described (28). Briefly, Sf9 cells were infected with XPA virus and the cellular pellet was lysed by dounce homogenization in buffer A containing 50 mM Tris, 100 mM NaCl, 0.1% (v/v) Triton X-100, 10% (v/v) glycerol, and 10 mM BME, along with a protease inhibitor cocktail. Following sonication, imidazole was added to 1 mM to the cellular extract and then loaded onto a 2 mL nickel-NTA agarose column. Bound protein was eluted in buffer A with 80 mM imidazole and protein containing fractions identified using Bradford analysis. Protein containing fractions were then pooled and loaded directly onto a 2 mL heparin-Sepharose column. Protein was eluted using a gradient from 100 mM to 1 M NaCl in heparin buffer (50 mM Tris, pH 7.5, 1 mM EDTA,

10% (v/v) glycerol, and 1 mM DTT with protease inhibitor mix). Fractions containing XPA were identified using Bradford and SDS-PAGE analysis, pooled and dialyzed overnight in heparin buffer and stored at -80°C .

Fluorescence Polarization

Fluorescence polarization was performed on 30-mer single strand DNA and duplex DNA with or without a 1,2 d(GpG) cisplatin lesion (10 nM) in buffer containing 20 mM HEPES, 1 mM DTT, 0.01% (v/v) NP40, and 100 mM NaCl. An excitation wavelength of 495 nm and a slit width of 5 nm were used in addition to an emission wavelength of 535 nm and 10 nm slit width. An XPA concentration of 30 nM (ssDNA) or 250 nM (dsDNA) was used to result in ~70% binding of XPA to DNA. Putative XPA inhibitors were titrated (0–200 μM) and polarizations values read. Results are presented as the average and standard deviation of at least three independent experiments.

ELISA

ELISA assays were performed by binding 200 fmol per well of biotinylated 60 base pair single-strand DNA in blocking buffer (2% (v/v) BSA TBS-Tween) overnight on streptavidin coated plates (Roche Applied Science). Inhibition of protein binding was examined by incubating 10 ng of purified XPA with increasing concentrations of compound in 150 μL reactions containing 5% (v/v) DMSO and blocking buffer for 10 minutes at room temperature. Following incubation, 100 μL of each reaction was added to separate wells, in duplicate, and incubated for 1 hour. The amount of XPA bound to DNA was analyzed by incubating 50 μL of goat anti-XPA antibody (Santa Cruz Biotechnology) (1:1000 in blocking buffer) in each well for 30 minutes with rocking. Following incubation with primary antibody, wells were washed 3×5 minutes with blocking buffer followed by the addition of goat anti-rabbit secondary antibody (Biorad) (1:2500 in blocking buffer) for 30 minutes. Wells were then washed as described above and TMB-ELISA reagent (Thermo Scientific) was added to each well. Kinetic reads were performed using a SpectraMax M5 spectrophotometer (Molecular Devices) for 20 minutes at 30 second intervals at wavelengths 490 and 370 nm. The maximum Abs sec^{-1} absorbance value was determined for each sample and represented as a percentage as of the absorbance value obtained for the control treated sample. The averages represent the results of duplicate samples from 4 independent experiments. The IC_{50} and standard deviation were calculated by fitting the curve to a standard 2-parameter hyperbolic curve. ELISA assays for the cisplatin damaged and undamaged double strand substrates were performed as described above, however, 10 and 50 ng of XPA were used, respectively.

Molecular modeling of XPA with small molecules

XPA interactions with small molecules were viewed using Pymol (DeLano Scientific) using cartoon and surface interaction views. Potential amino acid interactions were determined based on proximity to each compound as revealed by docking analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Recognition of DNA damage via the NER pathway

Recognition of DNA damage via TC_NER proceeds via RNA pol II encountering the lesion in complex with TFIIH. The addition of XPA and RPA completes the recognition process. In GG-NER, the XPA-Rad23B complex encounters the damage site followed by XPA-RPA and TFIIH after which XPC dissociates from the DNA. Following both processes, the structure specific nucleases XPF-ERCC1 and XPG catalyze incision 5' and 3' of the lesion indicated by the arrowheads. The damaged DNA strand is depicted in red, the undamaged strand in blue and the cisplatin lesion in green. The transcribed mRNA is depicted in magenta.

Figure 2. XPA-minimal DNA binding domain

Model of XPA MBD highlighting the region of the protein targeted for *in silico* screening. The targeted cleft is depicted in green and the image rotated 90 degrees.

Figure 3. *In vitro* analysis and chemical structures of XPA inhibitors

A) Analysis of *in vitro* inhibitory activity against XPA-DNA binding of compounds identified from the *in silico* screen by fluorescence polarization was performed as described in Methods and Materials. B, C and D, Chemical structures of SMIs identified in the *in silico* screen are depicted with the common structural features colored red.

Figure 4. XPA Inhibitors Reduce XPA-DNA Complex Formation *in vitro*

X80 (circle), X57 (square) and X60 (open triangle) were added in increasing concentrations to fluorescein-labeled single-strand DNA (Panel A), cisplatin damaged duplex DNA (Panel B) and undamaged duplex DNA (Panel C). Fluorescence polarization was monitored and DNA binding determined as described in Methods and Materials. The data are presented as the reduction in DNA binding compared to an untreated control and represent the mean and SD from three independent experiments.

Figure 5. X57 and X60 do not inhibit XPA-DNA binding as assessed in a modified ELISA

The indicated concentrations of X57 (white bars) and X60 (black bars) were incubated with 10 ng XPA for 10 minutes and binding to single strand DNA assessed by a modified ELISA as described in Methods and Materials (Panel A). X80 was preincubated with 10 ng XPA and analyzed for binding to single strand DNA (Panel B). 50 ng of XPA was analyzed for binding to undamaged duplex DNA (panel C) and 10 ng for analysis of binding to a duplex DNA containing a single 1,2-dGpG cisplatin lesions (panel D). The mean percentage of XPA bound compared to DMSO control and standard deviation from at least 3 independent experiments is presented.

Figure 6. Variable core structure support XPA inhibition

XPA inhibitor activity of X126 and X056 were assessed by FP analysis using a single strand DNA substrate. Fluorescence polarization was monitored and DNA binding determined as described in Methods and Materials. The data are presented as the reduction in DNA binding compared to an untreated control and represent the mean and SD from three independent experiments. Chemical structures of the two inhibitors are presented.

Figure 7. Docking analysis of SMI interactions with XPA

The docked X80 orientation and conformation in two XPA structures. A) X80 in 1XPA is shown in purple in ball-and-stick. B) X80 docked in 1D4U structure with best score is shown in blue in stick representation. The X80 orientation and conformation in 1XPA is also shown in 1D4U (purple) for comparison. C) Modeling X57 and X60 in the 1XPA MBD. Lys 141 and 179 are within interacting distance to the benzoic acid from each compound. D) Docking results with X80 reveals interactions of the furanyl benzoic acid with lys 137. E) Analysis of X801, X802 and X803 which each contain a single benzoic acid reorient within the MBD cleft to maintain an interaction with lys 137. F) Rotation of panel C 90°.

Table 1

Calculated IC₅₀ values for TDRL-X80 on Single, Double, and Cisplatin-Damaged DNA as Determined by Fluorescence Polarization and ELISA Analysis.

DNA Substrate	IC ₅₀ values (μ M)	
	Fluorescence Polarization	ELISA
single strand	18 $\pm$ 11	21 $\pm$ 3
ds-unplatinated	20 $\pm$ 10	39 $\pm$ 14
ds-platinated	29 $\pm$ 16	28 $\pm$ 8

* Inhibition values were obtained as described in Methods and Materials.

Table 2*In vitro* analysis of activity of X80 derivatives on XPA-DNA binding.

TDRL-	R1	R2	IC ₅₀ [*]
X801			>100 μM
X802			>100 μM
X803			75 μM

* Inhibition values were obtained using the modified ELISA assay on a single stranded 60 based DNA.

Table 3

Docked grid scores in 1xpa and 1d4u conformation.

TDRL-	Docking Grid Score (kcal/mol)	
	1XPA	1D4U
X57	-37	-36
X60	-37	-34
X80	-47	-43