Evaluating the Therapeutic Potential of a Non-Natural Nucleotide That Inhibits Human Ribonucleotide Reductase

Md. Faiz Ahmad¹, Qun Wan¹, Shalini Jha³, Edward Motea¹, Anthony Berdis¹, and Chris Dealwis¹,²,³

Abstract

Human ribonucleotide reductase (hRR) is the key enzyme involved in de novo dNTP synthesis and thus represents an important therapeutic target against hyperproliferative diseases, most notably cancer. The purpose of this study was to evaluate the ability of non-natural indolyl-2'-deoxyribose triphosphates to inhibit the activity of hRR. The structural similarities of these analogues with dATP predicted that they would inhibit hRR activity by binding to its allosteric sites. In silico analysis and in vitro characterization identified one particular analogue designated as 5-nitro-indolyl-2'-deoxyribose triphosphate (5-NITP) that inhibits hRR. 5-NITP binding to hRR was determined by isothermal titration calorimetry. X-ray crystal structure of 5-NITP bound to RR1 was determined. Cell-based studies showed the anti-cancer effects of the corresponding non-natural nucleoside against leukemia cells. 5-NITP binds to hRR with micromolar affinity. Binding does not induce hexamerization of hRR1 like dATP, the native allosteric inhibitor of hRR that binds with high affinity to the A-site. The X-ray crystal structure of Saccharomyces cerevisiae RR1-5-NITP (ScRR1-5-NITP) complex determined to 2.3 Å resolution shows that 5-NITP does not bind to the A-site but rather at the S-site. Regardless, 5-nitro-indolyl-2'-deoxynucleoside (5-NitR) produces cytostatic and cytotoxic effects against human leukemia cells by altering cell-cycle progression. Our studies provide useful insights toward developing new inhibitors with improved potency and efficacy against hRR. Mol Cancer Ther; 11(10); 1–10. ©2012 AACR.

Introduction

Ribonucleotide reductase (RR) is the sole enzyme that catalyzes the reduction of ribonucleoside diphosphates of adenine, guanine, uridine, and cytosine to their corresponding deoxyribose form. RR is a multi-subunit enzyme consisting of a large α (RR1) and a small β (RR2) subunit (1). RR1 contains 2 allosteric sites and a catalytic site, whereas the RR2 subunit houses a free radical required for catalysis (1). There are 4 classes of RR that are classified on the basis of free radical chemistry (2). All eukaryotic RR belong to class I that use a tyrosyl free radical (3, 4). RR1 contains a specificity site (S-site) that upon binding of the nucleoside triphosphates ATP/ dATP, TTP, or dGTP dictates the selection of CDP/UDP, GDP, and ADP substrates, respectively, for their conversion to the corresponding deoxyribose forms at the catalytic C-site (ref. 5; Fig. 1A). In addition, the N-terminus of RR1 contains a 4-helical bundle ATP-binding cone called the activity site (A-site; ref. 6). Binding of ATP at the A-site activates RR whereas the binding of dATP at this site inhibits RR activity (5).

While the abovementioned selection rules (5) are important for maintaining a balanced nucleotide pool, the molecular basis for this selection remained undefined for many years. However, insights into the intricate mechanism were made clear by several crystallographic studies (7–10). These studies show that a polypeptide chain called loop 2 (residues 285–296) connects the specificity site to the catalytic site (7–10). In the AMPPNP structure of ScRR1, loop 2 acts as a steric gate that blocks substrate binding at the C-site. This is in agreement with biochemical data showing that hRR activity is only 10% in the absence of nucleotide binding at the S-site (11, 12). When a nucleoside triphosphate binds at the S-site, loop 2 shifts away from the C-site toward the S-site to create space for substrate binding at the C-site (7). The nucleoside triphosphate that binds at this S-site show extensive interactions with residues 255 to 270 of loop 1 (Fig. 1A).

Several studies have shown that oligomerization of eukaryotic RR1 is required for regulation by the activator, ATP, and the inactivator, dATP (11–15). RR1 forms hexamers at physiologic concentrations of 3 mmol/L ATP and 20 to 50 μmol/L dATP. The dATP-induced hexamers are shown to be inactive whereas dimers retain activity (15). The latter was shown by the discovery of the D16R human and yeast RR1 mutants that form dATP-induced dimers but not hexamers, which retain wild-type-like...
activity (15). The dATP-bound ScRR1 structure revealed that the first 18 residues of the A-site are at the hexamer interface, providing an elegant model for dATP-induced hexamerization. It appears that ATP-induced hexamers have a different packing arrangement from the dATP-induced hexamers explaining how one can be active whereas the other inactive (15). A recent study by Stubbe and colleagues showed that the anti-cancer drug clofarabine, a dATP analogue, also causes hRR1 to hexamerize and further highlight the importance of oligomerization as a way to modulate hRR activity (16).

The crucial role of RR during replication makes it an important anti-viral and anti-cancer target (17–19). RR has 4 druggable sites on hRR1. These include the specificity site (S-site), the activity site (A-site), the catalytic site (C-site), and a peptide-binding site (P-site; refs. 20, 21; see Fig. 1A). Nucleoside analogues such as clofarabine and gemcitabine are important anti-cancer agents that can inhibit hRR activity by targeting the allosteric and catalytic sites of the enzyme (16, 22–24). In addition, fludarabine and cladribine are clinically used drugs and their metabolites target the hRR activity (25–27). In our previous structural study, we defined the molecular interactions between gemcitabine diphosphate interactions and the ScRR1 C-site (23). However, gemcitabine can also inhibit hRR through a mechanism that involves altering the oligomeric state of hRR. In this case, recent biochemical studies showed that gemcitabine diphosphate targets the hRR1 hexamer and inactivates it through a covalent modification (24).

In this current study, we investigate the ability of non-natural nucleotides to function as hRR1 inhibitors. We chose 5-substituted indolyl-2′-deoxynucleoside triphosphates (5-NIdR) as these analogues mimic the size and shape of dATP (Fig. 1E). In addition, previous studies showed that these analogues function as effective inhibitors of hRR1.
surrogates for dATP during the misreplication of damaged DNA (28). In this study, we examined whether these non-natural nucleotides can interact with the various druggable sites on hRR1 to generate anti-cancer effects. In silico screening identified 5-NITP as a potential lead candidate that can interact favorably with the A- and S-site of hRR1. We provide biochemical evidence that 5-NITP is a moderate inhibitor of hRR1. In addition, the corresponding non-natural nucleoside produces cytostatic effects against Jurkat cells, consistent with a mechanism involving the inhibition of hRR activity inside a cancer cell. The data from combined functional and structural studies illustrate how non-natural nucleotides can be rationally designed to inhibit key chemotherapeutic targets. Furthermore, a structural study provides insight into the design of additional non-natural analogues that possess improved selectivity and affinity as inhibitors of RR.

Materials and Methods

Compound synthesis

5-NldR and 5-NITP were synthesized and characterized as previously described (28).

Docking non-natural nucleotides into hRR1

In silico docking of the non-natural nucleotide library was conducted using Surflex dock module (29) integrated in Sybyl8.1.1. Non-natural nucleotides were docked against the crystal structure of hRR1 in complex with TTP and dATP bound at the S- and A-allosteric sites, respectively (15). The docked hits were scored using docking function and a consensus scoring function that averages score from many scoring functions (C-score). The docking function takes into account a linear combination of non-linear functions of atomic surface distances between proteins and ligand, steric, polar, entropic, and solvation effects (29).

Expression and purification of hRR1, hRR2, and ScRR1

hRR1 and hRR2 were expressed and purified as described in the work of Fairman and colleagues (15). ScRR1 was expressed and purified as described previously (7, 23). Briefly, the RR1 subunit of both hRR and ScRR are purified using peptide affinity chromatography. The small subunits of hRR and ScRR were purified using Ni- affinity chromatography. The iron was loaded onto the small subunit of hRR and ScRR using the procedures outlined previously (15).

IC50 determination

The activity of hRR was determined using in vitro 14C-ADP reduction assays as previously described (14, 23). Briefly, the buffer solution (50 mmol/L HEPES at pH 7.6, 5% (v/v) glycerol, 0.1 mol/L KCl) and the hRR2 protein solution were brought inside a glove box under deoxygenated conditions. A total of 5 equivalents of Fe(II) per hRR2 dimer from FeNH4SO4 based on Ferozine assay was added to the protein solution and incubated at 4°C in the glove box. The protein solution was removed from the glove box and the O2-saturated buffer was added. Excess iron was removed by S200 10/300 size exclusion chromatography. To determine the specific activity of hRR1, the reaction mixture contained 0.3 μmol/L hRR1 and 2.1 μmol/L hRR2 in an activity assay buffer of 50 mmol/L HEPES, pH 7.6, 15 mmol/L MgCl2, 1 mmol/L EDTA, 100 mmol/L/KCl, 5 mmol/L dithiothreitol (DTT), 3 mmol/L ATP, 100 μmol/L/dGTP, and 1 mmol/L 14C-ADP (~3,000 cpm/nmol). The reaction mixture was pre-incubated for 3 minutes at 37°C, and 30 μL aliquots were sampled at fixed time intervals after reaction initiation. Reactions were quenched by immersion in a boiling water bath, cooling, and treatment with alkaline phosphatase. Product 14C-dADP that formed during the reaction was separated from substrate 14C-ADP using boronate affinity chromatography (24). The amount of 14C-dADP formed was quantified by liquid scintillation counting using a Beckman LS6500 liquid scintillation counter. The IC50 was determined by using the specific activity of hRR1 at varying 5-NITP concentrations and determining the concentration of 5-NITP at 50% activity.

Multi-angle light scattering analysis of hRR1 bound with 5-NITP

Multi-angle light scattering (MALS) experiments were carried out immediately following size-exclusion chromatography (SEC) by online measurement of static light scattering (mini DAWN TREOS, Wyatt Technology), differential refractive index (dRI, Optilab rEX, Wyatt Technology) at a wavelength of 685 nm and ultraviolet absorbance at a wavelength of 280 nm (Dionex ultimate 3000 variable wavelength detector). A ProSEC 300S, 250 × 4.6 mm2 SEC column was connected upstream of the MALS-RI detectors and used to fractionate the injected sample. The SEC-MALS-RI system as a whole was validated using BSA (Sigma-Aldrich). Samples (20 μL) were injected onto an analytic SEC column ProSEC 300S, 250 × 4.6 mm2 (Varian, Inc). Before sample injection, the column was equilibrated at a flow rate of 0.3 mL/min in 50 mmol/L Tris, pH 7.6, 5 mmol/L MgCl2, 100 mmol/L/KCl, and 20 μmol/L 5-NITP. The chromatograms and resultant molecular weight data were analyzed using the Astra 5.3 software from the Wyatt Corporation.

Isothermal titration calorimetry

5-NITP binding to hRR1 subunit was measured by isothermal titration calorimetry (ITC) using a VT-ITC200 instrument (Microcal 432 Inc.). Aliquots (1.0 μL; except the first injection used 0.5 μL) of 1.0 mmol/L 5-NITP in buffer A [50 mmol/L Tris buffer, pH 7.9, containing 5 mmol/L MgCl2, 5 mmol/L DTT, 5% (v/v) glycerol] were injected into the cell containing 13 μmol/L hRR1 in buffer A at 25°C. The blank titration for hRR1 was conducted in buffer A at 25°C. The integrated heat of injection after correcting for the blank buffer was used to fit the 2-site sequential binding model using Microcal Origin 7.0 (Microcal Inc.). The observed binding constants were used to calculate the
Gibbs free energy relationship ($\Delta G$) using $\Delta G = -RT\ln K_{obs}$. $\Delta S$ and $-T\Delta S$ were calculated from $\Delta G$ using the Gibbs free energy equation, $\Delta G = \Delta H - T\Delta S$.

**Crystallization**

ScRR1 was crystallized as previously described (7). The ScRR1-NITP complex was obtained by powder soaking for 2 hours. We have found through experience with the ScRR1 system that the powder soaking method is the least harmful to crystals rather than soaking them in a different buffer from the mother liquor. After transferring the soaked crystals into the cryoprotectant solution [0.1 mol/L sodium acetate at pH 6.5, 25% PEG 3350, 0.2 mol/L ammonium sulfate, 20% (v/v) glycerol], the crystals were flash-frozen in liquid nitrogen for data collection.

**Data collection**

Data for the ScRR1-NITP crystal at 2.3 Å resolution belonging to space groups P21212 were collected at the GMCA 23ID-D beam line at the Advanced Photon Source at 100 K and were processed using HKL2000 (30). We also screened hRR1-5-NITP crystals at NE-CAT 24ID-E beam line.

**Structure determination, refinement, and analysis**

The structure of the ScRR1-NITP crystal was solved by the difference Fourier method. The model used was PDB ID 2CVV without any ligand, ion, or water. To decrease the difference Fourier peak, we used the phenix software suite (31). Model building was interspersed with refinement using Coot (32) and Phenix, respectively. Figures were generated using Pymol (33). Contacts between 5-NITP and ScRR1 were analyzed using Contact in CCP4i (34). Solvent accessible areas of the substrates in ScRR1 and hRR1 were analyzed using Areamol (35) incorporated in CCP4i.

**Cell culture procedures**

Jurkat cells [American Type Culture Collection (ATCC)] were cultured in a humidified atmosphere of 5% CO$_2$ at 37°C. Cells were maintained in Cellgro formulated RPMI-1640 supplemented with 10% heat-inactivated FBS, 5% l-glutamine, and 2.5% penicillin/streptomycin antibiotic. Cells were routinely propagated and used for experiments in logarithmic phase.

**Cell proliferation assays**

All the cell lines were authenticated by ATCC. Jurkat cells were obtained from the ATCC. Jurkat cells were maintained in RPMI-1640 media supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, 0.25 µg/mL amphotericin B, and 10% FBS and incubated at 37°C with 5% CO$_2$. Cells were seeded at a population density of approximately 200,000 cell/mL and treated with variable concentrations of non-natural nucleoside (0.1–100 µg/mL) for up to 72 hours. Viability was assessed via trypan blue staining and counting the number of viable (clear) versus nonviable (blue) cells under a microscope.

**Cell-cycle analyses**

Cells were grown at a density of 200,000/mL. 5-NIdR (100 mg/mL) was added for time periods varying from 1 to 3 days. Cells were harvested by centrifugation. The supernatant was removed and then washed with PBS. After aspiration of PBS, 500 µL of 70% ethanol was added and cells were incubated on ice for 15 minutes followed by centrifugation and the removal of ethanol. One milliliter of propidium iodide (PI) staining solution (10 mL of 0.1 Triton X-100/PBS, 0.4 mL of 500 µg/mL of PL, and 2 mg/mL of DNase-free RNase) was added to the cell suspension, placed on ice for 30 minutes, and then analyzed using a Beckman Coulter XL flow cytometer with a red filter.

**Accession numbers**

Atomic coordinates and structures factors have been deposited in the Protein Data Bank with the ID code: 3RSR.

**Results**

**In silico screening of non-natural nucleosides**

Several non-natural nucleoside analogues illustrated in Fig. 1E and the library reported in (36) were screened using the in silico docking program, surflex doc. On the basis of these studies show that 5-NITP is an excellent candidate that could bind to both the S- and A-site of hRR.

**ITC study of 5-NITP binding and IC$_{50}$ determination to hRR1**

To experimentally validate in silico findings, we used ITC to measure the dissociation constants for 5-NITP binding to hRR1. When 5-NITP was injected into the buffer [50 mmol/L HEPES at pH 7.0 containing 5 mmol/L MgCl$_2$, 5 mmol/L DTT, 5% (v/v) glycerol] alone, a relatively small exothermic heat change was observed (data not shown). A similar titration experiment with hRR1 resulted in large endothermic heat changes exhibiting characteristic binding isotherms (Fig. 2). The heat changes at various molar ratios of 5-NITP added to hRR1 can be best fit to a 2-binding site model that yields a $K_{d1}$ of 44 µmol/L and a $K_{d2}$ of 5 mmol/L. These results indicate that 5-NITP binds hRR1 at a high- and low-affinity binding site. The isotherm profile of 5-NITP binding to wild-type hRR1 shows a high enthalpy change and a positive entropic change, suggesting that binding of this non-natural nucleotide is driven primarily by enthalpy with small entropic contributions.

The IC$_{50}$ value for 5-NITP was determined by an in vitro activity assay using $^{14}$C-ADP or $^{3}$H-CDP as the substrates (15, 24). The wild-type hRR activity under hRR1 limiting conditions was as previously reported (15). Using ADP as the diphosphate substrate, the concentration of 5-NITP required to inhibit 50% hRR activity is 170 ± 5 µmol/L. Surprisingly, 5-NITP did not inhibit hRR activity when...
We next analyzed the effects of 5-NIdR on cell-cycle progression after 3 days post treatment using PI staining to measure cellular DNA content. The histogram of Jurkat cells treated with DMSO (Fig. 3B) shows a standard cell-cycle distribution for asynchronous cells as the vast majority of cells exist at G₁ (59% ± 2%) and S-phase (34% ± 2%) whereas a significantly smaller population exists at G₂–M (7% ± 1%). Treatment with 100 µg/mL of 5-NIdR produces 2 important effects (Fig. 3C). First, there is an increase in the percentage of sub-G₁ cells compared with those treated with DMSO. This increase is consistent with the cytotoxic effects of the non-natural nucleoside described above for the viability studies. Second, treatment with 5-NIdR also produces significant alterations in cell-cycle progression. In particular, cells treated with the non-natural nucleoside show significant accumulation at S-phase (45% ± 2%) with a concomitant decrease at G₁ (50% ± 1%) whereas a minimal decrease in populations at G₂–M (5% ± 1%; Fig. 3C). The increase is S-phase cells combined with a decrease in G₁ is consistent with a mechanism for inhibiting DNA synthesis; perhaps by reducing the availability of dNTPs caused by RR inhibition by 5-NIdR or its various phosphorylated metabolites.

Oligomeric state of hRR in the absence and presence of 5-NITP

The results of the docking studies combined with ITC data suggest that 5-NITP binds both at the S- and A-sites of hRR1. This is possible as 5-NITP is a dATP analogue. To further investigate whether 5-NITP binds to the A-site of hRR1, we carried out MALs experiments to determine the oligomeric state of hRR1 in the presence of 5-NITP. Because 5-NITP is a dATP analogue, it may bind at the A-site and thus induce hexamer formation, which has been documented with dATP. MALs determines the molecular weight of molecules via a method that is independent of molecular mass reference standards, column calibration, and assumptions of molecular shape (38). In addition, SEC-MALs separates mixtures of oligomers and measures the absolute molecular weight of an oligomer in elution fractions. As such, it provides an ideal technique to define the oligomeric state of hRR in the absence and presence of different nucleotide analogues. The results of this study reveal that the eluted fraction in the presence of 5-NITP corresponds to the dimer of hRR1 with a molecular weight of 176 kDa (Fig. 4). Exponentially growing Jurkat cells were treated with dimethyl sulfoxide (DMSO; vehicle control) or 2 fixed concentrations of 5-NIdR (50 or 100 µg/mL) for time periods of up to 3 days. Figure 3A provides representative time courses for the number of viable (green) versus nonviable (black) cells in the absence and presence of either 50 or 100 µg/mL 5-NIdR. Direct comparison of these time courses shows that treatment with 100 µg/mL of 5-NIdR produces a cytostatic effect as the number of viable cells is reduced by approximately 65% compared with cells treated with DMSO. In addition, 5-NIdR generates a weak cytotoxic effect due to the small yet reproducible increase in the number of nonviable cells.

Cell-based studies to evaluate the anti-cancer effects of 5-NIdR

We measured the cytostatic and/or cytotoxic effects of the corresponding nucleoside, 5-NIdR, against Jurkat cells. Jurkat cells are an attractive model for testing new anticancer agents as they display resistance to many existing chemotherapeutic agents (37). Exponentially growing Jurkat cells are an attractive model for testing new anticancer agents because they display resistance to many existing chemotherapeutic agents (37). We measured the cytostatic and/or cytotoxic effects of 5-NIdR against Jurkat cells. The results of the docking studies combined with ITC data suggest that 5-NITP binds both at the S- and A-sites of hRR1. This is possible as 5-NITP is a dATP analogue. To further investigate whether 5-NITP binds to the A-site of hRR1, we carried out MALs experiments to determine the oligomeric state of hRR1 in the presence of 5-NITP. Because 5-NITP is a dATP analogue, it may bind at the A-site and thus induce hexamer formation, which has been documented with dATP. MALs determines the molecular weight of molecules via a method that is independent of molecular mass reference standards, column calibration, and assumptions of molecular shape (38). In addition, SEC-MALs separates mixtures of oligomers and measures the absolute molecular weight of an oligomer in elution fractions. As such, it provides an ideal technique to define the oligomeric state of hRR in the absence and presence of different nucleotide analogues. The results of this study reveal that the eluted fraction in the presence of 5-NITP corresponds to the dimer of hRR1 with a molecular weight of 176 kDa (Fig. 4A). This contrasts the hexamer that is observed using dATP (14). It is important to mention here that our studies used 20 and 40 µM 5-NITP. Direct comparison of these time courses shows that treatment with 100 µg/mL of 5-NIdR produces a cytostatic effect as the number of viable cells is reduced by approximately 65% compared with cells treated with DMSO. In addition, 5-NIdR generates a weak cytotoxic effect due to the small yet reproducible increase in the number of nonviable cells.

X-ray crystallography of 5-NITP binding to RR1

To further understand the molecular basis for hRR1 inhibition by 5-NITP, we attempted to cocrystallize the hRR1–5-NITP complex. Unfortunately, we were unable to obtain an atomic resolution structure, as co-crystals of the hRR1–5-NITP complex diffused to a low resolution of 8 A. However, we previously showed that the enzyme for

CDP was used as the substrate (data not shown). This difference in inhibitory effects is consistent with the higher binding affinity of 5-NITP for the S-site as opposed to the A-site.

Figure 2. ITC profile of 5-NITP binding to the hRR1 subunit. The binding isotherm was obtained as described in Materials and Methods with correction for heat of dilution at 25°C. 5-NITP binding was derived from the nonlinear least-square fit of the isotherm. The isotherm profile of 5-NITP could be best fitted to the 2-site sequential binding model.
S. cerevisiae, ScRR1, has the most conserved structure compared with hRR1 (15). Human and yeast RR1 share 66% sequence identity and 83% sequence similarity. Furthermore, hRR1 shares structural homology with ScRR1, with an r.m.s. deviation of 0.8 Å. As such, we carried out soaking experiments using the previously reported ScRR1 crystals (7). Although previous attempts to cocrystallize other complexes have failed, it should be noted that ScRR1 cocrystallizes with TTP, which can be then subjected to soaking experiments. Here, we show that the ScRR1–5-NITP complex crystals diffracted to 2.3 Å resolutions, which allowed for structural determinations. The structure was refined to an acceptable range of $R$ and $R_{free}$ values with good geometrical parameters (Table 1). The 2$F_o$–$F_c$ difference map (Fig. 1B) clearly shows electron density for 5-NITP bound at the S-site. In addition, the $F_o$–$F_c$ omit maps show electron density for 5-NITP binding at the S-site (data not shown). In this structure, 5-NITP adopts a 2'-endo conformation as previously observed with the deoxyribonucleotides bound at the S-site (8, 14). The ScRR1–5-NITP complex structure superimposes with ScRR1–AMPPNP and hRR1–dATP complexed structures with RMSD of Cα atoms of 0.45 and 0.57 Å, respectively.

The interactions of 5-NITP bound at the S-site of ScRR1 are shown in Fig. 1D. 5-NITP interacts mainly with loop 1 (residues 255–270) and to a lesser extent with residue 285 of loop 2 (residues 285–296). Although the nitro group of 5-NITP does not make any H-bonds, it is involved in an ion–pair interaction with K243. This likely accounts for the enthalpic contributions determined in the ITC analysis (vide supra). In addition, the indole ring of 5-NITP makes van der Waals contacts with V286, K243, and T265. The 3'-OH of deoxyribose forms a strong H-bond (2.5 Å) with the carboxylic side chain of D226. As previously reported, we observe an Mg$^{2+}$ ion that coordinates the α- and γ-phosphate of 5-NITP. The presence of the Mg$^{2+}$ versus water was confirmed by comparing B-factors after refinement. Mg$^{2+}$ always refined with the lower B-factor, suggesting that the electron density peak corresponded to Mg$^{2+}$ rather than water. The Mg$^{2+}$ ion is octahedrally coordinated by 3 solvent molecules and the α- and γ-phosphates. The Nδ atom of K243 forms bifurcated salt bridges with...
the α- and β-phosphates. The α-phosphate forms 3 H-bonds with three solvent molecules. Two of these solvent molecules form several H-bonds with hRR1 at the S-site (Fig. 1D). The guanidinium side chain of R256 forms 2 salt bridges with the γ-phosphate. Overall, 5-NITP forms 12 H-bonds, 3 salt bridges, and 360 van der Waals contacts with residues and bound water molecules composing the S-site (Fig. 1D and Supplementary Table S1).

**Discussion**

This report describes the *in vitro* and *in vivo* characterization of a unique non-natural nucleoside analogue as an anti-cancer agent that inhibits the activity of hRR. Results from *in silico* docking studies of a library of non-natural nucleotide analogues identified one analogue, 5-NITP, that was predicted to bind both at the S- and A-site. ITC data show that 5-NITP binds one binding site on hRR1 with micromolar affinity and at another site with low millimolar affinity. In a recent study, we showed that a key to hRR inhibition by the negative regulator dATP is the ability of the natural nucleotide to induce the protein into an inactive hexamer (15). In an independent study, the

dATP analogue, clofarabine, was shown to inhibit hRR by inducing hexamer formation with hRR1 (16). This result further underscores the importance of developing chemical entities that inhibit hRR activity by modulating its oligomerization state (16). The results of our MALD studies reveal that 5-NITP, unlike dATP, does not induce hexamer formation in hRR1 at 20 to 40 μmol/L concentrations. We assume that the A-site of hRR1 is low affinity site for 5-NITP binding as the non-natural nucleotide is unable to hexamerize hRR1 when its concentration is maintained at 40 μmol/L (concentration for high-affinity binding site). In contrast, dATP was shown to hexamerize hRR1 at a concentration approximately equal to the KD for the A-site (15). The inability of 5-NITP to induce hexamerization of hRR is likely due to the extremely poor affinity (KD ~ 5 mmol/L) for 5-NITP binding at the A-site. Instead, the non-natural nucleotide induces dimer formation.

**Table 1. Data collection and refinement statistics**

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Figure 4. A. MALD analysis of 5 NITP interactions with hRR1. Oligomeric status of hRR1 was determined using Wyatt TRESOS MALD as described in Materials and Methods. The chromatograms and resultant molecular weight data were analyzed using the Astra 5.3 software from the Wyatt Corporation. B. Size exclusion chromatographic analysis of hRR1 in complex with 5-NITP. The calibration with molecular weight standards are shown in the inset.
(Fig. 4), which is consistent with previous reports, indicating that nucleotide triphosphates bind at the S-site located on the dimer interface (10). Finally, the crystal structure of the ScRR1–5-NITP complex confirms that the non-natural nucleotide binds at the S-site.

Previous mechanistic studies of 5-NITP have shown that the nitro moiety and the indole scaffold play important roles toward modulating the binding to various biologic targets. For example, 5-NITP functions as an excellent surrogate for dATP as a polymerase substrate during translesion DNA synthesis. In this case, the hydrophobic nature of the nitro moiety coupled with its extensive pi-electron density enhances its base stacking potential and allows for optimal insertion opposite DNA lesions such as the non-instructional abasic site (29). In addition, 5-NITP can function as a surrogate for ATP by inhibiting the activity of the bacteriophage T4 clamp loading complex, gp44/62, needed for assembly of the DNA replication complex. In this case, the nitro moiety plays multiple roles in initial ground state binding to the active site of the clamp loader. While the hydrophobic nature of nitro group is important, more favorable electrostatic interactions are formed between this moiety and an active site arginine residue. In addition, the indole ring interacts with an active site phenylalanine through of π–π electron stacking interactions. The structural data obtained here with 5-NITP bound to ScRR1 also highlight the importance of π–π stacking interactions. In particular, the conformation of the indole ring can be superposed onto the adenine ring of dATP bound to hRR1 by an approximate 90° rotation (ref. 15; Fig. 5A). It should be noted that we were able to do such comparison confidently, as previously we have shown that the S-site of ScRR1 is very similar to hRR1 (15). The relative orientation of the indole ring with respect to the adenine is forced to adopt this conformation as to avoid steric clashes with Y285 present on loop 2 (Fig. 5B). Similar clashes with loop 2 are observed when comparing the structures of ScRR1–5-NITP

**Figure 5.** Comparison of 5-NITP binding with dATP and AMPPNP. A, stereo figure of the mode of binding of 5-NITP (cyan) with dATP (yellow). B, stereo figure of the modeling of 5-NITP (cyan) bound at the hRR1 S-site (The model of 5-NITP adopting the dATP conformation is shown in green.) C, stereo figure shows the negative impact of the nitrate group (cyan) on Loop2 (yellow).
with ScRR1–AMPPNP (data not shown). In fact, the nitro group is likely to clash with residues 287 to 290 of loop 2, which results in the disorder of loop 2 in the electron density map of the ScRR1–5-NITP. Modeling studies of 5-NITP bound to hRR1 show that the interactions made to the S-site of ScRR1 are conserved (Fig. 5C).

The affinity of 5-NITP binding to the S-site of hRR1 compared with dATP is 75 times weaker (ref. 9; Fig. 2). One of the reasons for the poor affinity of 5-NITP is due to unfavorable electrostatic interactions between the nitro group and the carbonyl oxygen of K243. It appears that these unfavorable contacts are not accommodated well by the rearrangement of the S-site. 5-NITP forms less H-bonds and less ion–pair interactions than dATP bound at the S-site (Supplementary Table S1). 5-NITP has more surface accessible area (96 Å²) than dATP (76 Å²) when bound at the S-site. We attribute the loss of affinity of 5-NITP compared with dATP due to unfavorable electrostatic interactions, less H-bonds, less ion–pair interactions, and less surface area buried at the S-site.

Our data support a mechanism in which the inhibition of hRR1 by 5-NITP involves binding at the S-site as opposed to the A-site. This results in the disruption of the allosteric regulation of hRR as an effector nucleotide triphosphate binding at the S-site, which is required for substrate selection. This is clear as our data show that 5-NITP inhibits ADP reduction but does not inhibit CDP reduction. This difference in inhibitory effects indicates that 5-NITP must compete with the effector nucleotide triphosphate, dGTP, to disrupt the selection rules (5). It should be emphasized that adenine analogues binding at the S-site will not select for the ADP substrate. However, it is possible that 5-NITP inhibits secondary targets that are involved in DNA synthesis, which may explain its efficacy against cancer. Future studies will characterize compounds with amino substitutions on the indole ring. Moreover, it is now becoming evident that a good hRR inhibitor must have the ability to hexamerize hRR1 similar to dATP or clofarabine. Clearly 5-NITP is unable to hexamerize hRR1 at 20 to 40 µmol/L, the cellular concentration of dATP, hence explaining its moderate inhibitory potency. We propose that the ability to induce the formation of hRR1 hexamers can be used as a good indicator of a highly potent hRR inhibitor, a factor that should be implemented in inhibitor design.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: M.F. Ahmad, A. Berdis, C. Dealwis
Development of methodology: M.F. Ahmad, C. Dealwis
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M.F. Ahmad, Q. Wan, S. Jha, E. Motea, A. Berdis
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M.F. Ahmad, Q. Wan, S. Jha, A. Berdis
Writing, review, and/or revision of the manuscript: M.F. Ahmad, Q. Wan, S. Jha, E. Motea, A. Berdis, C. Dealwis
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): E. Motea
Study supervision: C. Dealwis

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