Mechanism of Action of an Imidopiperidine Inhibitor of Human Polynucleotide Kinase/Phosphatase*  

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The small molecule, 2-(1-hydroxyundecyl)-1-(4-nitrophenylamino)-6-phenyl-6,7a-dihydro-1H-pyrrolo[3,4-b]pyridine-5,7(2H,4aH)-dione (A12B4C3), is a potent inhibitor of the phosphatase activity of human polynucleotide kinase/phosphatase (PNKP) in vitro. Kinetic analysis revealed that A12B4C3 acts as a noncompetitive inhibitor, and this was confirmed by fluorescence quenching, which showed that the inhibitor can form a ternary complex with PNKP and a DNA substrate, i.e. A12B4C3 does not prevent DNA from binding to the phosphatase DNA binding site. Conformational analysis using circular dichroism, UV difference spectroscopy, and fluorescence resonance energy transfer all indicate that A12B4C3 disrupts the secondary structure of PNKP. Investigation of the potential site of binding of A12B4C3 to PNKP using site-directed mutagenesis pointed to interaction between Trp402 of PNKP and the inhibitor. Cellular studies revealed that A12B4C3 sensitizes A549 human lung cancer cells to the topoisomerase I poison, camptothecin, but not the topoisomerase II poison, etoposide, in a manner similar to small interfering RNA against PNKP. A12B4C3 also inhibits the repair of DNA single and double strand breaks following exposure of cells to ionizing radiation, but does not inhibit two other key strand-break repair enzymes, DNA polymerase β or DNA ligase III, providing additional evidence that PNKP is the cellular target of the inhibitor.  

The successful repair of DNA strand breaks is crucial to ensure the stability and survival of the cell. In the absence of correct and efficient repair, cells can accumulate mutations or undergo cell death. Several repair pathways are responsible for handling various classes of DNA lesions, such as damaged bases and single and double strand breaks, which can be generated by endogenous and exogenous genotoxic agents. In mammalian cells, polynucleotide kinase/phosphatase (PNKP) plays a role in the base excision/single strand repair repair pathway and the nonhomologous end-joining double strand-break repair pathway (1–5) because of its function in restoring the chemistry of strand breaks to a form suitable for DNA polymerases and ligases to complete the repair process, i.e. 3’-hydroxyl and 5’-phosphate termini. PNKP catalyzes the phosphorylation of 5’-OH termini and dephosphorylation of 3’-phosphate termini (6, 7). In the single strand-break repair (SSBR) pathway, PNKP acts in concert with XRCC1, DNA polymerase β (Pol β), and DNA ligase III (1, 5, 8). At double strand breaks, PNKP is dependent on DNA-dependent protein kinase catalytic subunit and XRCC4 (3, 9), which are components of the nonhomologous end-joining pathway. In addition, PNKP has been implicated in the repair of damaged termini generated by the topoisomerase I inhibitor camptothecin (4, 10), which forms a “dead end” complex in which topoisomerase I remains covalently attached to a 3’-phosphate group, thereby preventing topoisomerase I from resealing the DNA strand after the incision step (11). The inhibited enzyme can be cleaved from the phosphate group by the action of tyrosyl-DNA phosphodiesterase, leaving a break with 3’-phosphate and 5’-hydroxyl termini, which requires the subsequent action of PNKP (10). In contrast, PNKP does not appear to play a role in the DNA repair of topo II-dead end complexes generated by treatment with etoposide (4).  

Cancer cells in which PNKP expression has been depleted by targeted shRNA display a noticeable decrease in cell survival when exposed to a number of genotoxic agents, including ionizing radiation, camptothecin, and the alkylating agent, methyl methanesulfonate (4). This suggests that PNKP plays an important role in countering exogenous DNA damage, and it was proposed that PNKP may be a suitable target for small molecule inhibitors to enhance the toxicity of genotoxic therapeutic agents, especially ionizing radiation and topoisomerase I poisons (12, 13). We have recently identified a series of compounds from a library of polysubstituted imidopiperidines that inhibit the 3’-phosphate activity of PNKP (14). The most potent of these inhibitors, 2-(1-hydroxyundecyl)-1-(4-nitrophenylamino)-6-phenyl-6,7a-dihydro-1H-pyrrolo[3,4-b]pyridine-5,7(2H,4aH)-dione (A12B4C3) (Fig. 1), was shown to be non-toxic to A549 human lung cancer and MDA-MB-231 human breast cancer cells at 1 μM, but increased the sensitivity of these cells to ionizing radiation to almost the same extent as stable shRNA-mediated PNKP depletion (14). On the other hand, A12B4C3 failed to enhance the sensitivity of the shRNA-
mediated PNKP-depleted cells, indicating that PNKP is probably the major cellular target for the inhibitor.

This study describes the further characterization of the inhibitor at the cellular and molecular levels. We have compared its chemosensitizing properties on cells treated with topoisomerase I and II inhibitors to further substantiate the likelihood that PNKP is the cellular target for A12B4C3. At the molecular level, we have examined the influence of A12B4C3 on strand-break repair and identified its mechanism of inhibition. Finally, we have obtained information regarding the perturbation of PNKP by A12B4C3 and the site of interaction of the protein with the inhibitor.

MATERIALS AND METHODS

Reagents—The imidopiperidine A12B4C3 was resynthesized and purified as described (15). Recombinant human PNKP was purified as described previously (16) and stored in 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM MgCl₂, 0.5 mM dithiothreitol. Histidine-tagged human DNA ligase III cloned in pET28b was expressed in bacterial cells and purified on a Ni²⁺-charged His-Bind resin (Novagen) as described by the manufacturer. Human Pol β was kindly provided by Dr. Sam Wilson, NIEHS, National Institutes of Health, Research Triangle Park, NC. The His-tagged PNKP single mutants W402F and W331F were generated using the QuikChange Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA). Each reaction contained 50 ng of template pBluescript SK⁺/PNKP and 125 ng of sense and antisense primers. Eighteen PCR cycles were performed using an extension temperature of 65 °C. No further modifications were made to the protocol. The His-tagged mutant WFX402, in which all the tryptophans except 402 were replaced by phenylalanine, was generated with four rounds of mutagenesis using the QuikChange Multisite-directed Mutagenesis Kit (Stratagene, La Jolla, CA). For each reaction, 50 ng of pBluescript SK⁺/PNKP was used as a template together with 100 ng of the appropriate primer. The plasmids were sequenced on an ABI 310 genetic analyzer. The PNKP mutants were cloned into pET16b (Novagen/EMD Chemicals Inc., Gibbstown, NJ) using the BamHI and Xbal cleavage sites and transfected into Escherichia coli DE3(BL21) pLysS (Novagen) for expression. Recombinant His-tagged mutant PNKP proteins were purified from E. coli grown at 37 °C in 1–4 liters of LB medium supplemented with 50 μg of ampicillin. At an A₆₀₀ of 0.6, 0.1 mM isopropyl 1-thio-β-d-galactopyranoside (Sigma) was added and the culture was incubated for 24 h at 16 °C. The bacteria were spun down at 15,000 × g at 4 °C for 20 min. The pellet was resuspended in 40 ml of His-PNKP binding buffer (50 mM NaH₂PO₄, 250 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, pH 7.9). The solution was stirred on ice for 30 min in the presence of 30 mg of lysozyme and 4 mg of phenylmethylsulfonyl fluoride. The bacteria were disrupted by sonication and then spun at 15,000 × g for 20 min at 4 °C. The supernatant was loaded into a beaker with 4 ml of ProBond resin (Invitrogen) and mixed slowly for 1 h at 4 °C. Then the slurry was loaded into a column and the resin washed with 20 ml of 20 mM imidazole, before eluting the protein with 20 ml of 150 mM imidazole (4 × 5-ml fractions). The protein was concentrated using a 30-kDa cutoff Amicon Ultra-15 centrifugal filter (Millipore) and dialyzed with 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 5 mM MgCl₂. His-PNKP concentration was estimated by A₂₈₀, where 1.2 units were equivalent to 1.0 mg of protein.

Cells—A549 (human lung carcinoma cells) were obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured in a 1:1 mixture of Dulbecco’s modified Eagle’s medium/nutrient mixture F-12 supplemented with 10% fetal calf serum, penicillin (50 units/ml), streptomycin (50 μg/ml), l-glutamine (2 mM), non-essential amino acids (0.1 mM), and sodium pyruvate (1 mM), and maintained at 37 °C under 5% CO₂ in a humidified incubator. All culture supplies were purchased from Invitrogen. The generation of PNKP-depleted A549 cells (A549ΔPNKP) has been previously described (4).

Cytotoxicity Studies—The effect of PNKP inhibition by A12B4C3 on cellular survival following exposure to topoisomerase I and II poisons, camptothecin and etoposide, respectively, was measured in A549 and A549ΔPNKP cells using the clonogenic survival assay. Cells were seeded on 60-mm tissue culture plates at various cell densities to give between 100 and 1000 colonies per plate and returned to the incubator overnight to allow the cells to attach. For chemosensitization studies, the cells were incubated with or without 1 μM A12B4C3 for 2 h before exposure to different doses of camptothecin or etoposide (Sigma). After addition of topoisomerase poisons, cells were incubated for a further 24 h in the same medium and then washed twice with phosphate-buffered saline and incubated in fresh medium without the drug or PNKP inhibitor. Colonies were stained with crystal violet after 10 to 14 days and counted with an automated Colcount colony counter (Oxford Optronix, Oxford, UK).

DNA Strand-break Repair Measured by Single-cell Gel Electrophoresis—A549 and A549ΔPNKP cells were grown in 60-mm plates to form a confluent monolayer. A12B4C3 (1 μM) was added to the plates 2 h before irradiating cells. Cells were irradiated at 5 Gy (⁶⁰Co Gammacell; Atomic Energy of Canada Limited, Ottawa, Canada) and incubated at 37 °C for 0, 10, 30, 60, and 120 min. Single and double strand breaks were determined by single-cell gel electrophoresis under alkaline and neutral pH conditions, respectively, as previously described (4).

DNA Ligase III Assay—The substrate for this assay was composed of two short oligonucleotides, a 21-mer 5'-ATT-AAGAATGCCCACACCGCC-3' and a 5’-phosphorylated 24-mer 5’-pGGGCCCACACAGGTAATTGCCG-3’, annealed to a 45-mer 5’-GGCCAGCTAGTTGGTGGCCGCCGGTGGTGGGCATTGC-3’ (IDT, Coralville, IA). Prior to annealing, the 21-mer was radiolabeled with [γ-³²P]ATP (PerkinElmer Life Sciences) by incubation with 1
unit of T4 polynucleotide kinase (PNK) (New England Biolabs) for 20 min at 37 °C. After labeling, the T4 PNK was inactivated by boiling for 10 min. The oligonucleotides were annealed by adding equal molar concentrations of the oligonucleotides with 1/10 volume of 10× annealing buffer (100 mM Tris-HCl, pH 7.4, 1 mM NaCl, and 10 mM EDTA) and boiled for 10 min and then allowed to cool to room temperature. DNA ligase III reactions were set up as follows: 6 μl of distilled water, 2 μl of 10× ligase buffer (500 mM Tris-HCl, pH 7.5, 10 mM MgCl2, 1 mM ATP, and 10 mM dithiothreitol), 4 μl of A12B4C3 (to a final concentration of 1, 5, 12.5, 25, or 50 μM) or Me2SO, and 4 μl of human DNA ligase III (50 ng). The samples were incubated for 5 min at 30 °C and then 4 μl of annealed substrate (6.6 μM final concentration) was added and the samples incubated at 30 °C for a further 30 min, before the reactions were stopped by addition of 3× gel loading dye (Fisher Scientific, Ottawa, ON) and boiled for 10 min. Samples were loaded on a 12% polyacrylamide, 7 M urea sequencing gel and subjected to electrophoresis for 3 h in 1× TBE buffer. Gels were scanned with a Typhoon 9400 Variable Mode Imager (GE Healthcare, Little Chalfont, UK), and quantified using Image Quant 5.2 Software (GE Healthcare).

**DNA Polymerase β Assay**—The assay for Pol β activity utilized a similar substrate to that described for the ligase reaction except that a radiolabeled 20-mer replaced the 21-mer to generate a 1-nucleotide gap in place of a nick. Pol β reactions were set up as follows: 6 μl of distilled water, 2 μl of 10× polymerase buffer (500 mM Tris-HCl, pH 8.0, 100 mM MgCl2, 20 mM dithiothreitol, 200 mM NaCl, 10% glycerol), 2–4 μl of A12B4C3 (to a final concentration of 1, 5, 12.5, 25, or 50 μM) or Me2SO, and 2 μl of dNTP mixture (500 μM), and Pol β (100 ng). The sample was incubated for 5 min at 37 °C before addition of 4 μl of annealed substrate (6.6 μM final concentration). The samples were incubated at 37 °C for an additional 30 min and the reactions stopped by addition of 3× gel loading dye (Fisher Scientific) and boiled for 10 min. Samples were loaded on a 15% polyacrylamide, 7 M urea sequencing gel and the products separated by electrophoresis for 3 h in 1× TBE buffer. Gels were scanned with a Typhoon 9400 Variable Mode Imager and quantified using Image Quant 5.2 software. Failure of the 5’-phosphorylated 20-mer to undergo conversion to a 21-mer was indicative of inhibition of Pol β activity.

**Assay for 3’-Phosphatase Activity Based on the Release of Inorganic Phosphate (P_I)**—PNKP phosphatase reactions (20 μl total volume) were set up as follows: 1 μl of wild-type or mutant PNKP (100 ng), 2 μl of 10× phosphatase buffer (500 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, 1 mM spermidine, and 2.5 mM dithiothreitol), 2 μl of 1 mM 3’-phosphorylated 20-mer oligonucleotide, 15 μl of distilled H2O, and 1 μl of A12B4C3 to a final concentration of 1, 5, 12.5, 25, and 50 μM. The reactions were then transferred to a clear polystyrene colorimetric 384-well plate and incubated at 37 °C for 30 min. PiColorlock Gold reagent (Innova Biosciences Ltd., Cambridge, UK) was prepared shortly before use by addition of 1/100 volume of accelerator to PiColorlock Gold reagent as directed by the manufacturer. The gold mixture was then added to P_I-containing samples in a volume ratio of 1:4 and the samples incubated at room temperature for 30 min before the absorbance was read at 620 nm using a FLUOstar Optima® plate reader (BMG Labtech Inc., Durham, NC).

**Inhibition (Lineweaver-Burk Plot) Assay**—PNKP phosphatase reactions (20-μl total volume) were setup as follows: 1 μl of PNKP (100 ng), 2 μl of 10× phosphatase buffer (500 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, 1 mM spermidine, and 2.5 mM dithiothreitol), 2 μl of 0, 50, 100, 150, 200, 250, 300, or 500 mM 3’-phosphorylated 20-mer oligonucleotide, 15 μl of distilled H2O, and 1 μl of A12B4C3 to a final concentration of 5, 10, or 20 μM. The reactions were then transferred to a clear polystyrene colorimetric 384-well plate and incubated at 37 °C for 30 min. Phosphatase activity was measured by the PiColorLock Gold reagent as described above.

**UV Difference Spectroscopy**—UV difference spectra were recorded on a PerkinElmer Lambda 40 spectrophotometer over a wavelength range of 320–250 nm with 1-cm path length cells as described previously (17). To generate a UV difference spectrum, both sample and reference cells contained A12B4C3 (3 μM) and PNKP (0.5 mg/ml), but only the contents of the sample cell were mixed, thereby allowing the interaction between PNKP and A12B4C3 to proceed.

**Circular Dichroism Spectroscopy**—Circular dichroism measurements were performed in an Olis DSM 17CD spectropolarimeter (Bogart, GA) calibrated with a 0.06% solution of ammonium p-camphor-10-sulfonate. The temperature in the sample chamber was maintained at 5 °C. Each sample was scanned seven times and the experiments carried out as described previously (18). CD spectra were analyzed for secondary structures according to Chen et al. (19).

**Steady-state Fluorescence Spectroscopy**—Steady-state fluorescence spectra were measured at 25 °C on a PerkinElmer Life Sciences LS-55 spectrophotometer with 5-nm spectral resolution for excitation and emission as described in our earlier studies (8, 18). Protein fluorescence was excited at 295 nm, and fluorescence emission spectra were recorded in the 300–400-nm range; changes in fluorescence were usually monitored at the emission maximum (332 nm). Binding data were analyzed by nonlinear regression analysis using Prism (GraphPad Software Inc., La Jolla, CA). The oligonucleotide used for these studies (5’-biotin/ATT ACG AAT GCC CAC ACC GCPhos-3’) containing a biotinylated 5’ terminus and a 3’-phosphate terminus was obtained from IDT.

**Fluorescence Resonance Energy Transfer Measurements**—Nonradiative energy transfer between Trp residue 402 of mutant PNKP WFX402 and acrylcyanin (AC) was followed in these experiments. Addition of AC to the PNKP WFX402 mutant resulted in quenching of Trp402 fluorescence, and this was accompanied by the appearance of AC fluorescence around 500 nm. The estimation of molecular distances by intermolecular energy transfer between the emission transition dipole of a donor molecule and the absorption transition dipole of an appropriate acceptor is based on the theory of Förster (20). The rate of energy transfer from a specific donor to a specific acceptor (k_t) is given by,

\[
  k_t = \frac{(1/T_d)(R_o/R)^6}{R^6}
\]

(Eq. 1)

where T_d is the lifetime of the donor in the absence of the acceptor, R is the distance between the donor and acceptor, and
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FIGURE 2. Chemosensitization by A12B4C3. A, influence of A12B4C3 on the chemosensitivity of wild-type A549 cells and PNKP-depleted cells (A549ΔPNKP) to camptothecin. Cells were preincubated with 1 µM A12B4C3 for 2 h prior to addition of camptothecin and then maintained in the same medium for an additional 24 h. The medium was then replaced with fresh medium without the drug. The survival curves (± S.E.) are based on four independent sets of determinations. B, influence of A12B4C3 on the chemosensitivity of wild-type A549 cells and PNKP-depleted cells (A549ΔPNKP) to etoposide using identical conditions to those described in A. The survival curves (± S.E.) are based on four independent sets of determinations.

$R_0$ is a characteristic distance called the “Förster critical distance” at which the efficiency of transfer is 50%. $R_0$, which is dependent on spectral properties and the relative orientation of the donor-acceptor pair, can be evaluated from,

$$R_0 = \left(9.79 \times 10^5\right)\left(Jk^2Q_Dn^{-4}\right)^{1/6} \text{Å} \quad (\text{Eq. 2})$$

where $J$ is the spectral overlap integral of donor fluorescence and acceptor absorbance, $Q_D$ is the quantum efficiency of the donor, $n$ is the refractive index of the medium (taken to be 1.4), and $k^2$ is a dipole orientation factor. The value of $\frac{3}{2}$ for $k^2$ was used on the basis of the assumption of random rotation of the fluorophores (21). The overlap integral was approximated by Equation 3,

$$J = \sum F_D(\lambda)e_{\lambda}(\lambda)\lambda^4\Delta\lambda/\sum F_D(\lambda)\Delta\lambda \quad (\text{Eq. 3})$$

where $F_D(\lambda)$ is the corrected fluorescence of the donor, $e_{\lambda}(\lambda)$ is the molar extinction coefficient of the acceptor, and $\lambda$ is the wavelength in centimeters. The terms were summed over 2-nm intervals. The efficiency of energy transfer ($E$) was calculated from changes in fluorescence as follows,

$$E = 1 - (Q_{DA}/Q_D) \quad (\text{Eq. 4})$$

where $Q_D$ and $Q_{DA}$ are the quantum yields of the donor in the absence and presence of acceptor, respectively. The distances between the donor and acceptor were calculated from Equation 5.

$$E = \left(R_0/R\right)^6/[1 + (R_0/R)^6] \quad (\text{Eq. 5})$$

RESULTS

Cellular Chemosensitization by A12B4C3—We have previously shown that a non-toxic dose of A12B4C3 (1 µM) was able to sensitize cells to ionizing radiation. To examine the capacity of A12B4C3 to act as a chemosensitizer to topoisomerase inhibitors, clonogenic survival assays were performed to measure the response of A549 human lung cancer cells, and A549 cells depleted of PNKP by stable expression of shRNA for the response to etoposide (Fig. 2B) indicated that PNKP phosphatase inhibition did not sensitize cells to etoposide-induced DNA damage.

Influence of A12B4C3 on DNA Strand-break Repair—PNKP-depleted cells have previously been shown to have a reduced capacity for repair of radiation-induced single and double strand breaks. To determine whether A12B4C3 can mimic shRNA-mediated down-regulation of PNKP expression, we monitored the influence of A12B4C3 on strand-break repair in irradiated cells. For this experiment A549 cells were pretreated with 1 µM A12B4C3 for 2 h prior to 5-Gy irradiation and maintained in the presence of the inhibitor during the course of repair. SSBR was followed by single cell gel electrophoresis (comet assay) under alkaline conditions over 2 h and double strand-break repair (DSBR) by comet assay under neutral conditions over 24 h. For comparison, we also examined strand-break repair in A549ΔPNKP cells. The comets were visually scored using a 5-point scale that reflects the amount of undamaged DNA retained in the nucleus of the cell (head of the comet) to damaged DNA, which migrates out of the nucleus under electrophoresis (tail of the comet). Type 1 comets represent cells with undetectable levels of damage, whereas type 5 comets represent cells with very little intact DNA. The assessment of the comets indicated that the DNA in the A549 cells irradiated and incubated in the absence of inhibitor was approaching complete restoration to its initial (unirradiated) state by 2 h (Fig. 3), i.e. the majority of cells were scored as type 3–5 comets immediately after irradiation, but type 1 and 2 comets after 2 h. In the presence of A12B4C3, however, we still observed a high frequency of type 3–5 comets after 2 h, implying only limited SSBR. The low level of repair was comparable with that seen in the A549ΔPNKP in the absence of A12B4C3. When the inhibitor was applied to the A549ΔPNKP cells, the degree of repair appeared to be even more attenuated. A very similar set of results were observed for DSBR over 24 h (Fig. 4), with clear evidence for delayed repair in A12B4C3-exposed cells.
Influence of A12B4C3 on DNA Polymerase and DNA Ligase III—To determine whether A12B4C3 was reducing cellular SSBR by selectively inhibiting PNKP, we examined the ability of A12B4C3 to inhibit human DNA Pol and DNA ligase III, which are two other enzymes involved with PNKP in the SSBR complex (1). Double-stranded substrates carrying either a 1-nucleotide gap or a nick were used to test for Pol β and DNA ligase III activity, respectively. Pol β activity was measured on the basis of addition of a nucleotide at the site of the gap, whereas DNA ligase III activity was measured on the basis of linking the two short oligonucleotides flanking the nick to create a 45-mer. As shown in Fig. 5, compared with the positive control in the absence of inhibitor, there was no significant inhibition of incorporation of the missing nucleotide by Pol β or joining of the two shorter oligonucleotides by DNA ligase III when acting on their respective substrates, even at the highest concentration of A12B4C3 tested (50 μM). In contrast the human PNKP phosphatase activity is almost 100% inhibited by this concentration of A12B4C3 (14).

Mode of PNKP Phosphatase Inhibition by A12B4C3—The mode of enzyme inhibition by A12B4C3 was determined by a Lineweaver-Burk analysis of the substrate concentration dependence on the reaction. Phosphatase activity was determined using a previously described colorimetric assay in which the substrate is a 20-mer single-stranded oligonucleotide bearing a terminal 3′-phosphate group (14). To ascertain the mechanism of A12B4C3 inhibition, the assay was carried out using a fixed enzyme concentration while varying the concentration of the inhibitor (5, 10, and 20 μM). A plot of 1/S versus 1/V is shown in Fig. 6. The observed velocity V, which is a measure of the color development, decreased as the inhibitor concentration was increased, whereas the Km value remained the same. This type of response is the hallmark of a noncompetitive inhibitor.

Inhibitors that act noncompetitively do not impede the binding of the enzyme to its substrate and thus have the potential to form a ternary complex with the enzyme and substrate. We examined this possibility employing fluorescence spectroscopy, which also provided a means of obtaining binding constants of PNKP to the inhibitor and to a 3′-phosphorylated DNA substrate. Because binding of the inhibitor or the substrate to PNKP partially quenches the protein Trp fluorescence at 332 nm following
excitation at 295 nm, the binding affinity ($K_d$) can be determined by following fluorescence quenching as a function of ligand concentration (8). A representative plot of relative fluorescence intensity versus the concentration of A12B4C3 is shown in Fig. 7A (inset). Nonlinear regression analysis of the binding data revealed unimodal binding with a $K_d$ value of 0.37 $\pm$ 0.03 $\mu$M. We similarly measured the binding of PNKP to a single-stranded oligonucleotide bearing a phosphate at the 3’ terminus and biotin at the 5’ terminus. The latter group blocks the binding of PNKP to the 5’ terminus. We determined that PNKP bound this substrate with a $K_d$ value of 0.6 $\pm$ 0.05 $\mu$M (Fig. 7B).

Addition of the 3’-phosphorylated substrate to PNKP resulted in 20 $\pm$ 2% quenching of Trp fluorescence. Addition of A12B4C3 to PNKP in the presence of the substrate induced a further quenching of 17 $\pm$ 2% fluorescence, suggesting that the inhibitor was capable of binding to PNKP in the presence of its substrate. A similar result was obtained when the order of addition was changed. Thus, addition of A12B4C3 to PNKP resulted in 18 $\pm$ 2% quenching and the subsequent addition of the substrate yielded a further 20 $\pm$ 2% quenching. These data clearly demonstrate that the substrate and inhibitor can form a ternary complex with PNKP and confirm that the A12B4C3 acts as a noncompetitive inhibitor.

A12B4C3 Has No Effect on ATP Binding to PNKP—We have previously shown that A12B4C3 had a modest inhibitory effect on PNKP kinase activity. We therefore examined the influence of the inhibitor on the binding of ATP to PNKP by fluorescence titration (Fig. 8). The $K_d$ values for ATP binding in the absence and presence of A12B4C3 were 0.9 $\pm$ 0.1 and 1.0 $\pm$ 0.1 $\mu$M, respectively, indicating that the inhibitor exhibited no significant effect on ATP binding by the enzyme.
Effect of A12B4C3 Binding on PNKP Conformation—Circular dichroism, UV difference spectroscopy, steady-state fluorescence, and fluorescence resonance energy transfer measurements were carried out to study the effect of A12B4C3 binding on the secondary and tertiary structure of PNKP.

Circular Dichroism—Information concerning the secondary structure of PNKP was obtained from far-UV CD data, and a typical far-UV CD spectrum of PNKP is shown in Fig. 9. PNKP exhibited two large, negative CD bands centered around 209 and 219 nm, indicating the presence of $\alpha$-helical organization. The observed molar ellipticities, $[\theta]$, at these two wavelengths were $10,080 \pm 300$ and $9,200 \pm 300$ degrees cm$^2$ dmol$^{-1}$, respectively. The CD spectra were analyzed according to the method of Chen et al. (19). The protein possessed $\sim 28\%$ $\alpha$-helix and $\sim 37\%$ $\beta$-structure, and the remaining $\sim 35\%$ represented the random structure. It is evident from Fig. 9 that the addition of 2 $\mu$M A12B4C3 induced a conformational change in PNKP; the molar ellipticity values, $[\theta]$, at 209 and 219 nm were reduced to $-8,050 \pm 300$ and $-7,050 \pm 300$ degrees cm$^2$ dmol$^{-1}$, respectively. Analysis of the CD data indicated a decrease in $\alpha$-helical and $\beta$-structures accompanied by an increase in random structure. The calculated values were 22\% $\alpha$-helix and 30\% $\beta$-structure, whereas the random structure corresponded to 48\%.

Ultraviolet Difference Spectroscopy—The local environments of aromatic residues in a protein can affect its UV absorption spectrum. If the solvent polarity around an aromatic ring decreases, absorbance maximum will be shifted to longer wavelengths (red shift), and this will result in an increase in molar absorptivity (hyperchromic effect). On the other hand, if the solvent polarity around an aromatic ring increases, absorbance maximum will be shifted to shorter wavelengths (blue shift) and molar absorptivity will decrease (17, 22, 23). Fig. 10 shows the difference spectrum of PNKP when A12B4C3 was added to the sample cell and the contents were mixed, thereby allowing the interaction to proceed, whereas in the reference cell the protein and buffer containing A12B4C3 were not mixed. The concentration of protein and A12B4C3 was identical in both cells. The negative difference peak at 290 nm was characteristic of a blue shift of the tryptophan absorption band. The negative trough at 282 nm resulted from a blue shift of the tyrosine absorption band, and these blue shifts associated with tryptophan and tyrosine residues were interpreted as arising from an increased exposure of these aromatic groups to solvent (24).

Fluorescence Resonance Energy Transfer—AC, which reacts specifically with Cys groups in proteins, was used to label mutant PNKP WFX402 as described previously (8). PNKP
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WFX402-AC was functionally active when tested for its phosphatase and kinase activities and retained ~85% of its activities compared with unlabeled PNKP. The degree of labeling of PNKP WFX402 with AC was $1.4 \pm 0.2$ mol of AC/mol of PNKP WFX402. When the labeled protein was excited at 380 nm, the emission maximum occurred at 500 nm. The emission spectrum of PNKP, when excited at 295 nm, is shown in Fig. 11A. The excitation wavelength was 295 nm, and the concentration of AC added was 0.16 $\mu$mol. The excitation wavelength was 295 nm, and the concentration of AC added was 0.16 $\mu$mol. The excitation wavelength was 295 nm, and the concentration of AC added was 0.16 $\mu$mol. The excitation wavelength was 295 nm, and the concentration of AC added was 0.16 $\mu$mol.

WFX402-AC in the absence and presence of substrate, according to Equation 4. The transfer efficiencies were determined to be 0.25 for PNKP WFX402-AC and 0.31 for PNKP WFX402-AC in the presence of the 20-mer. The average distances ($R$ values) in Angstroms between Trp$^{402}$ and Cys residues were determined from the measured $E$ values according to Equation 5. The average distance $R$ for PNKP WFX402-AC was 32.90 Å, and this distance was reduced to 31.35 Å upon binding the 20-mer (Table 1). The $R$ value for the ternary complex involving PNKP WFX402-AC, 20-mer, and the A12B4C3 inhibitor was 31.92 Å, suggesting that a conformational change in the binary complex occurred when A12B4C3 bound to PNKP WFX402-AC in the presence of the oligonucleotide. In a follow up experiment, A12B4C3 was first added to PNKP WFX402-AC and subsequently the oligonucleotide was added to this binary complex. In this instance also, there was clear evidence for a ternary complex formation, implying that binding of the DNA substrate and A12B4C3 is mutually exclusive, i.e. they bind at different sites on PNKP.

Localization of A12B4C3 Interaction Site with PNKP—In an earlier study (8), we showed that the observed Trp fluorescence quenching caused by DNA binding results mainly from the perturbation of Trp$^{402}$. For this reason, we made mutants W402F and W331F in which Trp at positions 402 or 331 were replaced by Phe. All three mutants retained good 3'-phosphorylated activities. The observed inhibitory effects of A12B4C3 on these mutants are presented in Table 2. A12B4C3 was effective involving PNKP WFX402-AC, 20-mer, and the A12B4C3 inhibitor was 31.92 Å, suggesting that a conformational change in the binary complex occurred when A12B4C3 bound to PNKP WFX402-AC in the presence of the oligonucleotide. In a follow up experiment, A12B4C3 was first added to PNKP WFX402-AC and subsequently the oligonucleotide was added to this binary complex. In this instance also, there was clear evidence for a ternary complex formation, implying that binding of the DNA substrate and A12B4C3 is mutually exclusive, i.e. they bind at different sites on PNKP.

these conditions, the observed peak at 500 nm represented Trp-excited AC fluorescence and demonstrated energy transfer to AC.

Fig. 11B shows the emission spectrum of mutant PNKP WFX402 and the absorption spectrum of AC-labeled PNKP WFX402. There was substantial overlap of the absorbance and emission spectra. The overlap integral $J$, calculated according to Equation 3, was $2.81 \times 10^{-14}$ cm$^2$ M$^{-1}$. Assuming $k^2 = \frac{3}{4}, n = 1.4$, and the quantum yield of the donor emission (PNKP) in the absence of the acceptor equals 0.10, the Förster critical distance $R_0$ was 27.45 Å for PNKP WFX402-AC. The transfer efficiency was 0.25. The determined values of $E$ and $R_0$ were used to calculate $R$, the apparent average distance (32.90 Å) separating Trp residue 402 from the AC-labeled Cys residues (Table 1).

The emission spectrum of AC-labeled PNKP WFX402, excited at 295 nm, is shown in Fig. 11C. The observed fluorescence at 332 nm was due to Trp residue 402 and the fluorescence peak at 500 nm corresponds to Trp-sensitized AC fluorescence. Addition of a 3'-phosphorylated/5'-biotinylated 20-mer quenched the Trp fluorescence of PNKP WFX402-AC, suggesting an increase in transfer efficiency and providing evidence for a conformational change in PNKP WFX402-AC upon substrate binding. The effects on transfer efficiencies were measured by determining quantum yields of PNKP WFX402-AC in the absence and presence of substrate, according to Equation 4. The transfer efficiencies were determined to be 0.25 for PNKP WFX402-AC and 0.31 for PNKP WFX402-AC in the presence of the 20-mer. The average distances ($R$ values) in Angstroms between Trp$^{402}$ and Cys residues were determined from the measured $E$ values according to Equation 5. The average distance $R$ for PNKP WFX402-AC was 32.90 Å, and this distance was reduced to 31.35 Å upon binding the 20-mer (Table 1). The $R$ value for the ternary complex involving PNKP WFX402-AC, 20-mer, and the A12B4C3 inhibitor was 31.92 Å, suggesting that a conformational change in the binary complex occurred when A12B4C3 bound to PNKP WFX402-AC in the presence of the oligonucleotide. In a follow up experiment, A12B4C3 was first added to PNKP WFX402-AC and subsequently the oligonucleotide was added to this binary complex. In this instance also, there was clear evidence for a ternary complex formation, implying that binding of the DNA substrate and A12B4C3 is mutually exclusive, i.e. they bind at different sites on PNKP.

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FIGURE 11. A, fluorescence emission spectra of PNKP (■) and PNKP-AC (▲). The concentration of hPNKP was 0.2 $\mu$mol in 50 mM Tris, pH 7.5, 100 mM NaCl, and 5 mM MgCl$_2$. The concentration of AC added was 0.16 $\mu$mol. The excitation wavelength was 295 nm. B, overlap of the Trp emission spectrum of mutant PNKP WFX402 (■) excited at 295 nm with the absorption spectrum of AC-labeled PNKP WFX402 (▲). The right ordinate gives fluorescence intensities measured in arbitrary units; the left ordinate is calibrated in terms of the molar absorbance of the PNKP WFX402-AC complex. C, fluorescence emission spectra of PNKP WFX402-AC (upper) and with 1 $\mu$mol 20-mer 3'-phosphorylated oligonucleotide (lower). The excitation wavelength was 295 nm, and the concentration of the AC-labeled PNKP WFX402 was 0.2 $\mu$mol.

Because free AC does not fluoresce under these conditions, the observed peak at 500 nm represented Trp-excited AC fluorescence and demonstrated energy transfer to AC.

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TABLE 2
Effect of A12B4C3 binding on wild-type and mutant PNKP phosphatase activity and Trp fluorescence

| PNKP    | Phosphatase activity | Inhibition | Trp quenching |
|---------|----------------------|------------|---------------|
| Wild-type | 100                  | 100        | 18            |
| W331F   | 90                   | 95         | 17            |
| W402F   | 85                   | 6          | 17            |
| WFX402  | 85                   | 90         | 18            |

All measurements were carried out at 25 °C in 50 mM Tris, pH 7.5, 100 mM NaCl, and 5 mM MgCl₂.

**TABLE 1**

Fluorescence energy transfer parameters of AC-labeled PNKP-WFX402 mutant

|          | Overlap integral, *[^a^] (cm² Å⁻²) | Critical distance, *[^b^] Å | Efficiency of transfer, *[^b^] % | Distance between Trp and Cys, *[^b^] Å |
|----------|-----------------------------------|-----------------------------|----------------------------------|-------------------------------------|
| WFX402   | 2.82 × 10⁻¹⁴                      | 27.50                       | 25                               | 32.95                               |
| WFX402 + DNA | 2.79 × 10⁻¹⁴                      | 27.40                       | 31                               | 31.30                               |
| WFX402 + A12B4C3 | 2.76 × 10⁻¹⁴                      | 27.38                       | 28.5                             | 31.95                               |
| WFX402 + A12B4C3 | 2.74 × 10⁻¹⁴                      | 27.35                       | 22                               | 34.10                               |
| WFX402 + A12B4C3 + DNA | 2.75 × 10⁻¹⁴                      | 27.37                       | 30                               | 31.40                               |

[^a^]: Determined as described under "Materials and Methods."

[^b^]: Energy transfer efficiency was calculated from changes in fluorescence intensity. The relative errors in the R values are ± 0.1 Å.

DISCUSSION

We previously identified A12B4C3 from a library of polysubstituted imidopiperidines based on its ability to inhibit the phosphatase activity of PNKP in vitro (14). The compound sensitized wild-type A549 cells to ionizing radiation, but not PNKP-knockdown (A549 PNKP) cells, suggesting that PNKP is its cellular target. However, to confirm this, we further compared the properties of A12B4C3-treated cells to A549 PNKP cells, in particular their response to topoiso merase I and II poisons, camptothecin and etoposide. The 3’-phosphate and 5’-hydroxyl strand-break termini generated in camptothecin-treated cells require the remedial action of PNKP, unlike the termini generated by etoposide. A549 cells treated with A12B4C3, like A549 PNKP cells, responded in accord with the expectation that the inhibitor would sensitize the cells to camptothecin but not etoposide (Fig. 2). In addition, A12B4C3 did not sensitize the A549 PNKP cells to camptothecin (Fig. 2), providing evidence to substantiate PNKP as the cellular target for the inhibitor. Because PNKP depletion has been shown to slow the rates of SSBR and DSBR (4), we anticipated that the inhibitor would elicit the same response. Measurement of SSBR and DSBR by single cell gel electrophoresis following exposure of cells to ionizing radiation indicated that A12B4C3 does indeed slow strand rejoining in cells (Figs. 3 and 4), and the demonstration that the inhibitor has no effect on two other key SSBR proteins, DNA Pol β and DNA ligase III, strongly implicates PNKP inhibition as the mode of action of the compound.

Our biochemical and biophysical analyses clearly revealed that A12B4C3 acts as a noncompetitive inhibitor that allosterically regulates the phosphatase activity of human PNKP. This provides an explanation for our previous observations regarding the specificity of the inhibitor. The phosphatase domain in human PNKP encompasses residues 146 to 336 and has a fold typical of the haloacid dehydrogenase superfamily (6, 7, 25). DNA phosphatases from other species also belong to the haloacid dehydrogenase superfamily and have conserved residues that are involved in catalysis or in binding the Mg²⁺ and the phosphate moiety of the substrate and phosphoaspartate intermediate. The inhibitory effect of A12B4C3 when tested against a number of closely related phosphatases such as the PNKP enzymes isolated from bacteriophage T4, Schizosaccharomyces pombe, and mouse, which belong to the haloacid dehydro genase superfamily, exhibited strong specificity toward the mammalian PNKPs (14). It would seem less likely for a competitive inhibitor to show this degree of specificity.

A12B4C3 also inhibits the kinase activity of PNKP, albeit to a lesser degree than the phosphatase activity (14). The kinase (Phe₃⁴¹-Glu₅₂⁰) and the phosphatase domain (Gly₁⁴⁶-Glu₃₃⁷) together constitute the catalytic fragment, which carries out the enzymatic activities of PNKP (25). The kinase and phosphatase domains in the catalytic fragment contact one another by two short polypeptides: the intradomain linker (Leu₃₃₈-Ala₃₄⁰) and the C-terminal tail (Gln₅₁⁷-Glu₅₂¹), which interacts with the phosphatase domain. The individual catalytic domains need to be within the catalytic fragment to retain their functional activities and proper folding. The catalytic domains could not be separated by proteolysis (25). This intimate association between the kinase and phosphatase domains could explain why binding of A12B4C3 at or near Trp⁴⁰² in the kinase domain is able to inhibit both activities of human PNKP by inducing a conformational change in the protein.

A12B4C3 exhibited strong affinity (Kd = 0.37 ± 0.03 μM) for PNKP and the IC₅₀ value obtained was 0.06 μM (14). Despite being a noncompetitive inhibitor, the IC₅₀ value for A12B4C3 is comparable with the reported values for other inhibitors targeting DNA repair proteins. For example, SU11752, which selectively inhibits DNA-PK by competing with ATP, has an IC₅₀ value of 0.13 μM (26), and recently developed pyrrolocarbazole lactam-based inhibitors of poly(ADP-ribose) polymerase, which compete against NAD⁺, were found to have IC₅₀ values in the range of 0.02–0.1 μM (27).

In summary, we have further characterized the nature of the inhibition of PNKP by the imidopiperidine A12B4C3, showing it to be a tight-binding noncompetitive inhibitor that interacts with Trp⁴⁰². Because it sensitizes cells to camptothecin as well as ionizing radiation, A12B4C3, or derivatives of this compound, may have a potential clinical benefit in countering the
Inhibitor of Polynucleotide Kinase/Phosphatase

resistance of cancer cells to these agents. Data from this study will assist in the design of more potent derivatives of A12B4C3 and help define the protein binding pocket responsible for the interaction with PNKP.

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