Identification of a Small Molecule Inhibitor of the Human DNA Repair Enzyme Polynucleotide Kinase/Phosphatase

Gary K. Fresauch,1,2 Feridoun Karimi-Bushe,1,2 Agnieszka Ula czyk-Lesanko,3 Todd R. Mereniuk,1,2 Ashley Ahrens,1,2 Jonathan M. Koshy,1,2 Aghdass Rasouli-Nia,1,2 Phuwat Pasarj,1,2 Charles F.B. Holmes,4 Frauke Rininsland,5 Dennis G. Hall,3 and Michael Weinfeld1,2

1Experimental Oncology, Cross Cancer Institute, and Departments of 2Oncology, 3Chemistry, and 4Biochemistry, University of Alberta, Edmonton, Alberta, Canada; and 5QTL Biosystems, Santa Fe, New Mexico

Abstract

Human polynucleotide kinase/phosphatase (hPNKP) is a 57.1-kDa enzyme that phosphorylates DNA 5′-termini and dephosphorylates DNA 3′-termini. hPNKP is involved in both single- and double-strand break repair, and cells depleted of hPNKP show a marked sensitivity to ionizing radiation. Therefore, small molecule inhibitors of hPNKP should potentially increase the sensitivity of human tumors to γ-radiation. To identify small molecule inhibitors of hPNKP, we modified a novel fluorescence-based assay to measure the phosphatase activity of the protein, and screened a diverse library of over 200 polysubstituted piperidines. We identified five compounds that significantly inhibited hPNKP phosphatase activity. Further analysis revealed that one of these compounds, 2-(1-hydroxyundecyl)-1-(4-nitrophenylamino)-6-phenyl-6,7a-dihydro-1H-pyrrolo[3,4-b]pyridine-5,7(2H,4H)-dione (A12B4C3), was the most effective, with an IC50 of 0.06 μmol/L. When tested for its specificity, A12B4C3 displayed no inhibition of two well-known eukaryotic protein phosphatases, calcineurin and protein phosphatase-1, or APTX, which is required for the repair of damaged bases and single-strand breaks induced by many agents including ionizing radiation and alkylating agents (1, 3, 4), tyrosyl DNA-phosphodiesterase, which is required for the repair of strand breaks introduced by topoisomerase 1 inhibitors such as camptothecin and irinotecan (5), and ATM and DNA-PK, which regulate the response to DNA double-strand breaks (6, 7). Inhibitors of PARP are now in clinical trial (8).

Irradiation and other genotoxic agents often generate strand breaks with incompatible termini that must be processed for single- and double-strand break repair pathways to complete the repair. Among the frequently observed termini are 3′-phosphate and phosphoglycolate and 5′-hydroxyl groups (9–11). These lesions create a barrier for DNA polymerases and ligases to replace missing bases and seal the breaks because these enzymes have a strict requirement for the presence of a 3′-hydroxyl group and in addition DNA ligases require a 5′-phosphate group (12, 13). It is now clear that the major enzyme responsible for the phosphorylation of 5′-hydroxyl termini and dephosphorylation of 3′-phosphate termini in human cells is polynucleotide kinase/phosphatase (hPNKP; refs. 14, 15). In the single-strand break repair pathway, hPNKP acts in concert with XRCC1, DNA polymerase β, and DNA ligase III (16–18), whereas PNPK-mediated DNA end processing at double-strand breaks is a component of the nonhomologous end-joining pathway and is dependent on DNA-PKcs and XRCC4 (19–21). In addition to its role in the repair of strand breaks produced directly by genotoxic agents, hPNKP has been implicated in the repair of strand breaks produced by DNA glycosylases such as NEIL1 and NEIL2 (22, 23), and the topoisomerase I inhibitor camptothecin (24). Given the involvement of hPNKP in several repair pathways, it is not surprising that its down-regulation by RNAi-sensitized cells to a variety of genotoxic agents including ionizing radiation, camptothecin, methyl methanesulfonate, and hydrogen peroxide (25). It remains to be determined which of hPNKP’s activities, 5′-kinase or 3′-phosphatase (or both), is responsible for sensitization to each agent. The two activities are independent with each active site containing its own DNA binding domain (26), but the phosphatase reaction seems to proceed ahead of the kinase reaction (27).

It has recently been suggested that PNKP could be a potential target for small molecule inhibitors (2, 28). We report here the development of a fluorescence-based assay for screening chemical
libraries for inhibitors of the phosphatase activity of PNKP and its application in the screening of a library of drug-like polysubstituted piperidines. The compound scaffold in this library possesses a range of stable functionalities such as imide, hydroxyl, and basic amino groups that confer hydrogen bond donor/acceptor capabilities that could promote interaction with proteins (29). We have identified several compounds that inhibit hPNKP phosphatase activity and further show that one of these compounds sensitized cells to ionizing radiation. It thus has the potential to be a useful laboratory reagent for studying the role of hPNKP in the cellular response to ionizing radiation and other genotoxins, and act as the initial compound in the development of a drug targeting hPNKP in the course of cancer therapy.

Materials and Methods

Enzymes. Recombinant hPNKP was purified as described previously (15, 30) and stored in 50 mmol/L Tris-HCl (pH 7.4), 100 mmol/L NaCl, 5 mmol/L MgCl2, and 0.5 mmol/L DTT. Recombinant mouse PNKP was purified as previously described (26). Schizosaccharomyces pombe PNKP was purified as described previously (31). Phage T4 polynucleotide kinase was purchased from Roche Diagnostics. The catalytic subunit of human protein phosphatase-1γ (PP-1γ) was expressed in Escherichia coli and purified as previously described (32). Rat β-calcineurin protein was expressed in E. coli and purified as previously described (33).

Human recombinant aprataxin (APTX) protein with an NH2-terminal 6× His tag was expressed in BL21-Gold (DE3) E. coli–competent cells (Stratagene) using the QIAgene expression construct (Qiagen). A single colony of kanamycin-resistant E. coli was used to inoculate a 200-ml overnight culture in Luria-Bertani media containing 30 μg/ml kanamycin. Four 50-ml fractions of overnight culture were then subcultured into 4 × 1 l Luria-Bertani without kanamycin. Once the culture reached an absorbance of ~0.6 at 600 nm, protein expression was induced using 0.2 mmol/L isopropyl-1-thio-β-galactopyranoside (Sigma) at 37°C for 2 h. Cells were harvested by centrifugation at 10,000 rpm for 10 min at 4°C and resuspended in 40 ml buffer [50 mmol/L NaH2PO4, 250 mmol/L NaCl, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF; pH 7.9)]. The solution was then stirred on ice for 30 min in the presence of 30 mg lysozyme and 4 mg PMSF, and 1 μg/ml each of pepstatin and leupeptin. The bacteria were then sonicated 6 × 30 s allowing 30 s between intervals to cool down. The cell debris was then spun down at 15,000 rpm for 15 min at 4°C and the supernatant collected. The supernatant was then stirred on ice in the presence of 4 ml Probond resin (Invitrogen) for 1 h and then loaded onto a column. The resin was washed with 3 × 5 ml 20 mmol/L imidazole and 5-ml fractions were collected. Then, 25 ml of 150 mmol/L imidazole was loaded onto the column and 1-ml fractions were collected. Fractions were run on a 10% SDS-PAGE and stained with Coomasie Brilliant Blue R-250 (Invitrogen). Fractions (150 mmol/L) showing high concentrations and single bands were then combined and concentrated using a 30-kDa cutoff Amicon Ultra-15 centrifugal filter (Millipore) and dialyzed with 50 mmol/L Tris-HCl (pH 7.5), 100 mmol/L NaCl, and 5 mmol/L MgCl2. His-APTX concentration was then determined using the Bio-Rad Protein assay (Bio-Rad).

Cells. A549 (human lung carcinoma cells) and MDA-MB-231 (human breast adenocarcinoma cells) were obtained from the American Type Culture Collection. Cells were cultured in a 1:1 mixture of DMEM/mutrient mixture F-12 (DMEM/F-12) supplemented with 10% FCS, penicillin (50 U/ml), streptomycin (50 μg/ml), L-glutamine (2 mmol/L), nonessential amino acids (0.1 mmol/L), and sodium pyruvate (1 mmol/L), 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 has been previously described (25). The PNKP-depleted MDA-MB-231 cells were generated in a similar fashion except that the shRNA-expressing pSUPER vector used on this occasion (pSUPERneo, OligoEngine) also contained the cDNA for the G418 selectable marker.
Optimization of fluorescence quenching–based assay for PNKP 3’-phosphatase activity. We modified the Lightspeed assay developed originally for protein kinases and phosphatases by QTL Biosystems. This reagent is no longer produced or distributed by QTL Biosystems. The standard substrate used for this assay was a 20-mer oligonucleotide (5’TAMRA-AAT ACG AAT GCC CAC ACC GC-P-3’ labeled with 5-(6-carboxytetramethylrhodamine) (TAMRA) at the 5’-end and bearing a terminal 3’-phosphate (Integrated DNA Technologies). The TAMRA-labeled oligonucleotide lacking a 3’-phosphate served as a control. Four calibration solutions, consisting of 0%, 25%, 50%, and 100% 3’-phosphorylated oligonucleotide, were prepared by mixing the two oligonucleotides (i.e., 3’-phosphorylated and nonphosphorylated oligonucleotides) in respective proportions (0.5 μmol/L total oligonucleotide concentration). The assay was performed in 384-well white Optiplate microplates (PerkinElmer) in 70 mmol/L Tris-HCl (pH 7.4), 60 mmol/L MgCl2, 5 mmol/L MnCl2, 0.3% bovine serum albumin (BSA), and 0.09% sodium azide. Reaction buffer was prepared by adding 1 mmol/L DTT immediately before use. Five microliters of 3’-phosphatase substrate (final concentration 0.5 μmol/L) were used per well. In duplicate, 10 ng each concentration of hPNKP was added per well. Plates were incubated for 1 h at 37°C and then 15 μL of 1x sensor solution (provided by QTL) were added to each well and incubated for 30 min at room temperature. Fluorescence (485 nm excitation and 520 nm emission wavelengths) of each well was read in a FLUOstar Optima (BMG Labtech, Inc.). Data were analyzed using GraphPad Prism Software.

Acquisition and screening of the small molecule library. A previously described library of 244 polysubstituted piperidines (29) encompassing a diversity of functional groups and substituents was synthesized as described (29) and used for the screening. Small molecules were provided in powder form and were dissolved in 100% DMSO, and a final concentration of 100 μmol/L was added to each well and assays were performed as described above.

After obtaining an optimum calibration curve and enzyme concentration curve, we used a simplified form of the assay to test the library in a short time. Only one concentration of hPNKP, 50 ng, was tested and compared with the control well with no enzyme. The assay was conducted in the same way as described above with respect to oligonucleotides, buffer, controls, incubation lengths/temperatures, centrifugations, and sensor addition.

Assay for 3’-phosphatase activity based on the release of inorganic phosphate. hPNKP phosphatase reactions (20 μL total volume) were setup as follows: 1 μL hPNKP (100 ng), 2 μL 10x phosphatase buffer [500 mmol/L Tris-HCl (pH 7.4), 0.1 mmol/L EDTA, 1 mmol/L spermidine, and 2.5 mmol/L DTT], 15 μL distilled H2O, and 1 μL small molecule (varying concentrations) were incubated at 37°C for 5 min, and then 2 μL of 1 mmol/L 3’-P 20mer oligonucleotide was added (The oligonucleotide had the same sequence as that used in the fluorescence quenching assay, but without the TAMRA substituent). 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.) was prepared shortly before use by addition of 1/100 vol. of accelerator to PiColorlock Gold reagent as directed.

Figure 2. Chemical structures and names of the compounds found to inhibit hPNKP phosphatase activity.
by the manufacturer. The Gold mix was then added to inorganic phosphate–containing samples in a volume ratio of 1:4, and the samples were incubated at room temperature for 30 min before the absorbance was read at 620 nm using a FLUOstar Optima plate reader (BMG Labtech, Inc.).

Conventional radio-gel assay for hPNKP 3’-phosphatase activity. hPNKP phosphatase activity was determined by monitoring the removal of the 3’-phosphate from a [γ-32P]-labeled 20-mer oligonucleotide containing a 3’-phosphate (5’-ATT ACG AAT GCC CAC ACC GC-P-3’) as previously described (15). Briefly, the 5’-end of the oligomer was labeled by incubation with phage T4 phosphatase–free polynucleotide kinase (Roche Diagnostics) and [γ-32P]ATP (PerkinElmer). To the labeled oligomer, hPNKP that had been preincubated for 5 min at 37°C with varying concentration of small molecule inhibitor in phosphatase buffer was added, and the reaction was allowed to proceed for 20 min. The level of 3’-dephosphorylation was monitored by electrophoresis on a 12% polyacrylamide/7 mol/L urea sequencing gel for 3 h in 1× Tris-borate EDTA buffer. Gels were scanned with a Typhoon 9400 Variable Mode Imager (GE Healthcare), and quantified using Image Quant 5.2 Software (GE Healthcare).

PP-1cγ and calcineurin phosphatase inhibition assay. PP-1cγ and calcineurin activity was analyzed, using a colorimetric p-nitrophenol phosphate assay as previously described (34). Inhibition reactions were carried out in a 96-well microplate with a final volume of 60 μL containing 40 μL of p-nitrophenol phosphate assay buffer [50 mmol/L Tris (pH 7.4), 0.1 mmol/L EDTA, 30 mmol/L MgCl2, 0.5 mmol/L MnCl2, 1 mg/mL BSA, and 0.2% (v/v) mercaptoethanol], 0.03 μg PP-1cγ (specific activity of >30 units per mg) or a catalytically equivalent quantity of calcineurin and 10 μL of 0.5 mmol/L or 10 μL of 50 μmol/L A12B4C3 in DMSO, or control solvent. After a 10-min incubation at 37°C, 10 μL of 30 mmol/L p-nitrophenol phosphate was added to each well and incubated for an additional 60 and 45 min for PP-1cγ and calcineurin, respectively. The absorbance at 405 nm was measured using a SOFTmax 2.35 kinetic microplate reader (Molecular Devices).

DNA kinase assay. Kinase reactions (20 μL final volume) were set up as follows: 1 μg hPNKP was preincubated with increasing concentration of A12B4C3 in the kinase buffer [80 mmol/L sucinic acid (pH 5.5), 10 mmol/L MgCl2, and 1.0 mmol/L DTT] at 37°C for 5 min and then 20-mer oligonucleotide substrate (20 nmol) and 3.3 pmol of [γ-32P]ATP were added and the reaction mixture was incubated at 37°C for 20 min. The reaction was stopped by addition of an equal volume of DNA loading dye (90% formamide, 0.02% bromophenol blue, and 0.02% xylene cyanol in 1× TBE). Samples were boiled for 5 min and the products separated on a 12% polyacrylamide/8 mol/L urea gel. Gels were scanned with a Typhoon 9400 Variable Mode Imager (GE Healthcare), and quantified using Image Quant 5.2 Software (GE Healthcare).

Cell proliferation assay. To determine the effect of the inhibition of PNKP by small molecule inhibitors on cell proliferation, we used the CellTiter 96 AQeuous Non-Radioactive Cell Proliferation Assay (Promega), better known as the MTS assay. Approximately 2.5 × 104 A549 cells were plated in triplicate in a 96-well plate with different concentrations of A12B4C3 inhibitor. After 72 h, 20 μL of CellTiter 96 Aqueous One Solution...
Reagent was added to each well and cells were incubated for 4 more hours at 37°C. The absorbance recorded at 490 nm on a FLUostar Optima plate reader was used as a representation of the relative number of living cells in culture.

Cytotoxicity studies. The effect of hPNKP inhibition by A12B4C3 on cellular survival following exposure to ionizing radiation was measured in A549, A546PNKP, and MDA-MB-231 and MDA-MB-231hPNKP cells by clonogenic assays. Cells were seeded on 60-mm tissue culture plates at various concentrations to give between 100 to 1,000 colonies per plate and returned to the incubator overnight to allow the cells to attach. For radiosensitization studies, the cells were incubated with or without 1 μmol/L A12B4C3 for 2 h before irradiation and then exposed to increasing doses of γ-radiation (60Co Gammacell, Atomic Energy of Canada Limited). After irradiation, cells were incubated for a further 24 h in the same media and then washed twice with PBS and incubated in fresh media without the inhibitor. Colonies were stained with crystal violet after 10 to 14 d and counted with an automated Colcount colony counter (Oxford Optronix).

Results

Screening of the library by a fluorescence-based assay. The fluorescence-based phosphatase assay that we adapted was originally developed to monitor protein phosphatase activity (35). The assay involves a fluorescent sensor molecule coupled with trivalent metal ions that bind to phosphate groups. When the sensor is brought into close proximity with a phosphorylated and dye (TAMRA)-labeled substrate, superquenching of the sensor occurs. Removal of the phosphate leads to an elevation of fluorescence because the sensor is not brought close enough to TAMRA for its signal to be quenched. We modified the buffer conditions so that the internucleotide phosphate groups of a DNA substrate would not interfere strongly with the process of measuring the presence of a terminal phosphate group as shown by the standard curve of 0%, 25%, 50%, and 100% phosphorylated oligo solutions (Fig. 1A). We then determined the amount of hPNKP required for near complete dephosphorylation of the oligonucleotide by measuring the fluorescence signal as a function of hPNKP present in the reaction (Fig. 1B), and as a result, we chose 50 ng as the standard quantity of hPNKP for each reaction in the screen. Heat-inactivated hPNKP was used as a control. We screened a small molecule library consisting of over 200 polysubstituted piperidines (29) for their capacity to inhibit the phosphatase activity of hPNKP. Five of the compounds, A12B4C3, A1B4C3, A68B4C3, A26B11C2, and A39B1C2, were observed to cause significant inhibition as shown in Fig. 1C. Also shown are the data for three other compounds, A4B8C2, A28B3C1, and A24B12C3, as examples of the majority of compounds that failed to inhibit hPNKP. The formal names and chemical structures of the active compounds are shown in Fig. 2.

Confirmation of inhibition of PNKP phosphatase activity. A conventional radio-gel assay was used to verify the inhibition of hPNKP phosphatase activity by these small molecules. This assay shows a shift on an acrylamide sequencing gel that corresponds to 3'-phosphate removal from a 20-mer single-stranded oligonucleotide (36). We confirmed that all five of the positively identified compounds inhibited hPNKP phosphatase activity. We also examined a number of small molecules shown by the screening assay not to be inhibitors of hPNKP phosphatase activity, and they also failed to show inhibition by the radio-gel approach. Examples of the assay are shown in Fig. 3.

Inhibitory activity and specificity of A12B4C3. To further assess the activity of the five inhibitory molecules, we made use of a proprietary colorimetric reagent (PicoColorLock Gold) that measures release of inorganic phosphate (Pi). A drawback encountered with the fluorescence-based approach can be fluorescence quenching arising from direct interaction of the small molecules with the fluorescent sensor.

| Table 1. Inhibition by A12B4C3 of other related DNA phosphatases |
|-----------------|-----------------|-----------------|
| Protein         | Enzyme concentration | % Phosphatase activity |
|                 | No Inhibitor | 50 μmol/L | 12B4C3 |
| hPNKP           | 5 ng/μL | 100 ± 0.5   | 0 ± 1.3 |
| mPNKP           | 5 ng/μL | 96 ± 2.8     | 6 ± 2.6 |
| S. pombe PNKP   | 5 ng/μL | 94 ± 1.7     | 68 ± 5.2 |
| T4 PNK          | 0.05 U/μL | 92 ± 2.4  | 79.5 ± 3.8 |
| hAPTX           | 50 ng/μL | 70 ± 0.9      | 70 ± 1.2 |

**Figure 5.** Specificity of inhibition by A12B4C3. A, the influence of A12B4C3 on protein phosphatases was examined as described in Materials and Methods. No inhibition by A12B4C3 of PP-1 or calcineurin (CaN) was observed. In comparison, the potent PP-1 inhibitor microcystin LR effectively inhibited PP-1 at low nanomolar concentrations (12 nmol/L), as expected. B, dose-dependent inhibition of hPNKP DNA kinase activity by A12B4C3 measured by the transfer of radiolabeled phosphate from [γ-32P]ATP as described in Materials and Methods. Data for each figure were compiled from three independent assays for each activity measured. Columns, mean; bars, SEM.
Methods. The survival curves (was determined by the colony-forming assay as described in Materials and Methods. The media was then replaced with fresh media without the drug. Cytotoxicity was measured 2 h before irradiation and then maintained in the same media for a further 24 h.

PNKP-deficient cells (\textit{B,} influence of A12B4C3 on the radiosensitivity of wild-type A549 cells and MDA-MB-231 breast cancer cells to increasing concentrations of the compound and determination of cell proliferation as described in Materials and Methods. The data are drawn from three independent determinations \pm SEM. \textit{B,} influence of A12B4C3 on the radiosensitivity of wild-type A549 cells and PNKP-deficient cells (A549/PNKP). Cells were exposed to 1 \mu{\text{mol/L}} A12B4C3 2 h before irradiation and then maintained in the same media for a further 24 h. The media was then replaced with fresh media without the drug. Cytotoxicity was determined by the colony-forming assay as described in Materials and Methods. The survival curves (\pm SEM) are based on four independent sets of determinations. \textit{C,} influence of A12B4C3 on the radiosensitivity of wild-type MDA-MB-231 cells and PNKP-depleted MDA-MB-231 cells (MDA-MB-231(PNKP)) using identical conditions to those described in \textit{B.} The survival curves (\pm SEM) are based on five independent sets of determinations.

Figure 6. Radiosensitization by A12B4C3. \textit{A,} cytotoxicity of A12B4C3 alone measured by 72-h exposure of A549 lung cancer cells and MDA-MB-231 breast cancer cells to increasing concentrations of the compound and determination of cell proliferation as described in Materials and Methods. The data are drawn from three independent determinations \pm SEM. \textit{B,} influence of A12B4C3 on the radiosensitivity of wild-type A549 cells and PNKP-deficient cells (A549(PNKP)). Cells were exposed to 1 \mu{\text{mol/L}} A12B4C3 2 h before irradiation and then maintained in the same media for a further 24 h. The media was then replaced with fresh media without the drug. Cytotoxicity was determined by the colony-forming assay as described in Materials and Methods. The survival curves (\pm SEM) are based on four independent sets of determinations. \textit{C,} influence of A12B4C3 on the radiosensitivity of wild-type MDA-MB-231 cells and PNKP-depleted MDA-MB-231 cells (MDA-MB-231(PNKP)) using identical conditions to those described in \textit{B.} The survival curves (\pm SEM) are based on five independent sets of determinations.

Cancer Res 2009; 69: (19). October 1, 2009 7744 www.aacrjournals.org

Published OnlineFirst September 22, 2009; DOI: 10.1158/0008-5472.CAN-09-1805

molecule with the sensor agent. (Note in Fig. 1C the lower fluorescence signal of the sensor caused by exposure to some compounds in the absence of PNKP). This problem is avoided in the colorimetric assay, which measures the release of inorganic phosphate from a 20-mer 3'-P oligonucleotide based on the change in absorbance of malachite green in the presence of molybdate. Based on the standard curve obtained using 0%, 25%, 50%, and 100% phosphorylated substrates (Fig. 4A), we found that A12B4C3 was the most potent of the five PNKP inhibitors (Fig. 4B) and obtained an IC\textsubscript{50} dose of 0.06 \mu{\text{mol/L}} and near maximal inhibition with a concentration of 10 \mu{\text{mol/L}} (Fig. 4C). The curve was fitted to conform to a single site-specific binding with Hill slope. Deviation from the curve at high concentration of inhibitor most likely reflects the limitation of the assay when measuring low levels of released inorganic phosphate. The IC\textsubscript{50} value for the next most active inhibitor, A6B4C3, was determined and found to be somewhat higher (~0.3 \mu{\text{mol/L}}).

Specificity of A12B4C3. To determine the specificity of A12B4C3 for hPNKP phosphatase activity, we examined a number of closely related phosphatases such as the PNKP enzymes isolated from bacteriophage T4, \textit{Schizosaccharomyces pombe}, and mouse, as well as human APTX. We observed that 50 \mu{\text{mol/L}} A12B4C3 inhibited phage T4 and the \textit{S. pombe} PNKPs by \sim15% and \sim30%, respectively, compared with complete inhibition of hPNKP (Table 1). Not surprisingly, the compound significantly inhibited mouse PNKP (which shares \sim80% identity to hPNKP). We also tested whether A12B4C3 could inhibit APTX, which is another human DNA 3'-phosphatase (37). For this experiment, the oligonucleotide substrate was incubated with equal quantities of the two enzymes that were purified from bacteria on the same day. We observed that APTX has a robust phosphatase activity that was totally refractory to 50 \mu{\text{mol/L}} A12B4C3 (Table 1). We then broadened our examination to look at two well-known protein phosphatases, calcineurin and PP-1. Neither enzyme displayed any inhibition when treated with an A12B4C3 concentration as high as 83.3 \mu{\text{mol/L}} whereas the control inhibitor microcystin LR reduced the activity of PPI \sim65% (Fig. 5A).

Finally, the effect of A12B4C3 on the kinase activity of hPNKP was examined by quantifying the transfer of 32P-labeled phosphate from radiolabeled ATP to an oligonucleotide. As shown in Fig. 5B, there was a concentration-dependent increase in inhibition of the PNKP kinase activity up to \sim45% at 50 \mu{\text{mol/L}} A12B4C3. However, the inhibition of the kinase and phosphatase activities cannot be directly compared because the standard assay for kinase activity uses 10-fold more enzyme than the assay for phosphatase activity.

Cytotoxicity of A12B4C3 and cellular radiosensitization by A12B4C3. The data presented indicated that A12B4C3 is a potent inhibitor of hPNKP \textit{in vitro}. It was therefore important to assess the compound's effectiveness as a radiosensitizer. A12B4C3 was first tested for its inherent toxicity. Cytotoxicity was measured by cell proliferation assay after exposure of A549 human lung carcinoma cells and MDA-MB-231 breast adenocarcinoma cells to increasing doses of the compound for 72 hours (Fig. 6A). We observed a dose-dependent reduction in cell proliferation up to \sim50% at 100 \mu{\text{mol/L}} A12B4C3, which is close to the limit of solubility of the compound. No significant effect on cell proliferation was detected after exposure of either cell line to 1 \mu{\text{mol/L}} A12B4C3. The lack of cytotoxicity at this dose was confirmed by clonogenic survival assay following exposure to A12B4C3 up to 24 hours (data not shown). We then examined the capacity of A12B4C3 to act as a radiosensitizer. A549 cells were incubated with 1 \mu{\text{mol/L}} A12B4C3.
for 2 hours before irradiation and then maintained in the presence of the compound for a further 24 hours. The survival curves indicated that exposure to A12B4C3 almost doubled the radiosensitivity of A549 cells (Fig. 6B). This radiation response was nearly identical to that seen with cells depleted of PNKP by stable expression of shRNA (A549/PNKP). On the other hand, A12B4C3 failed to sensitize the PNKP-depleted cells. Similar data were obtained with wild-type and PNKP-depleted MDA-MB-231 breast cancer cells (Fig. 6C).

Discussion

In this report, we describe the initial steps toward the generation of a selective hPNKP inhibitor because our previous observations indicated that PNKP depletion, mediated by shRNA, sensitizes cells to ionizing radiation (25), and would therefore be an appropriate target for small molecule inhibition. PNKP possesses 5’-kinase and 3’phosphatase activity. For this study, we chose to target the latter activity, because 3’-phosphate termini are frequently generated by ionizing radiation (9, 11). This necessitated the development of a suitable screening assay for inhibitors of the phosphatase activity. Most fluorescence-based high throughput screening assays for phosphatase activity have been directed toward protein phosphatases and rely on immunodetection using antibodies to the phosphorylated peptide substrate. The superquenching assay, originally devised by Rininsland and colleagues (35), presented an alternative approach that depended on the presence of a phosphate group for chemical recognition. It required some optimization involving protonation of the substrate to enhance the influence of the terminal phosphomonoester group over the internucleotide phosphodiester groups of the DNA substrate. Using this protocol, a Z-factor of 0.68 was obtained, which is considered sufficient for identification of inhibitors in high throughput screens (38). The inhibitory activity of compounds identified by the superquenching assay could be corroborated by the conventional radio-gel assay and by the PiColorlock colorimetric assay.

The chemical library of polysubstituted piperidines proved a relatively rich source of inhibitory compounds. Noticeably, the three most active compounds contain a paranitrophenyl hydrazine substituent on the ring nitrogen of the six-membered piperidine ring, as well as an N-phenyl imide unit (Fig. 2). The importance of these substituents to the binding of the inhibitor to PNKP remains to be determined through further structure-activity relationship studies. The library features less chemical diversity at the position of the N-phenyl imide, which could be the target of future structure-activity optimization. A12B4C3 was clearly the most effective inhibitor of PNKP with an IC50 of 0.06 μmol/L compared with ~0.3 μmol/L for the next most active compound A6B4C3 (Fig. 4C). In addition to the paranitrophenyl hydrazine substituent, A12B4C3 also features a long hydrophobic alky1 chain, which may at least be partly responsible for the greater inhibitory activity displayed by this compound. However, as seen from the structures of the active compounds shown in Fig. 2, all the compounds have different substituents at this position, suggesting that this site may be less critical and consequently it could be a potential site for bioconjugation required in radiolabeling and mechanistic studies.

An important issue with all small molecule inhibitors is their specificity. We examined the response of a number of other phosphatases to A12B4C3. Phage T4 polynucleotide kinase and hPNKP share similar nucleic acid kinase and phosphatase activities. However, with the exception of the enzyme active sites, the proteins bear no recognizable homology (26, 28). The phosphatase domains of both proteins belong to the haloacid dehalogenase superfamily (26, 29, 40) with a conserved DxGDT motif, where the first Asp forms a covalent phospho-aspartate intermediate with the substrate. The fact that A12B4C3 poorly inhibited T4 polynucleotide kinase (Table 1) suggests that the small molecule does not directly interact with this conserved haloacid dehalogenase motif. The catalytic domain (phosphatase and kinase) of S. pombe PNKP, on the other hand, shares considerably more structural similarity with hPNKP than the T4 enzyme, with 127 identical residues, including the haloacid dehalogenase motif. The catalytic domain (phosphatase and kinase) of S. pombe PNKP, on the other hand, shares considerably more structural similarity with hPNKP than the T4 enzyme, with 127 identical residues, including the haloacid dehalogenase motif. Hence the strong inhibition of human and mouse PNKP (Table 1). Because A12B4C3 also inhibits the kinase activity of hPNKP (Fig. 5B), it is possible that the compound acts as a noncompetitive inhibitor disrupting the structure of the enzyme. Indeed, preliminary evidence suggests that this is the case.6

Given the low level of inhibition of related polynucleotide kinases/phosphatases, it is not surprising that A12B4C3 displayed no inhibition (Fig. 5A) for some of the two protein phosphatases tested, protein phosphatase 1 (PP-1) and calcineurin (protein phosphatase 2B), which are important members of the eukaryotic serine/threonine family involved in a broad range of signal transduction pathways (41). Of course, we cannot rule out the possibility that A12B4C3 interacts with other protein phosphatases or indeed other enzymes, but our tests for radiosensitization by A12B4C3 (Fig. 6B and C) indicated not only that the compound effectively sensitized wild-type cells to ionizing radiation, but also revealed that PNKP is most likely the cellular target for A12B4C3 in human cells because it failed to sensitize the PNKP-deficient cells.

In summary, we have made use of several analytic approaches to identify a set of polysubstituted piperidine molecules that inhibit mammalian PNKP in the μmol/L range and may prove to be useful tools in the study of DNA repair. The most potent of these compounds, A12B4C3, will serve as a useful lead compound for future development of potential clinical radiosensitizers.

Disclosure of Potential Conflicts of Interest

A provisional patent for the PNKP inhibitors has been submitted by M. Weinfield and D.G. Hall. The other authors disclosed no potential conflicts of interest.

Acknowledgments

Received 5/15/09; revised 7/27/09; accepted 8/6/09. Published OnlineFirst 9/22/09.

Grant support: Canadian Institutes of Health Research (CIHR) and Alberta Cancer Foundation (M. Weinfield); CIHR (Drug Development Initiative) and Natural Sciences and Engineering Research Council of Canada (D.G. Hall); and from CIHR (C.F.B. Holmes). J.M. Koshy was the recipient of a summer studentship from the Alberta Cancer Research Institute.

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We thank Mesfin Fanta for his technical assistance and Tamara Skene and Hue Anh Lui for technical assistance with protein phosphatase preparation, and Dr. Jean-Marie Grassot and Eric Pelletier for a larger scale resynthesis and purification of compound A12B4C3.

6 R.S. Mani, personal communication.


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