

Inhibition of Human Tyrosyl-DNA Phosphodiesterase by Aminoglycoside Antibiotics and Ribosome Inhibitors

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ABSTRACT

DNA topoisomerase I (Top1) is the target of camptothecin, and novel Top1 inhibitors are in development as anticancer agents. Top1 inhibitors damage DNA by trapping covalent complexes between the Top1 catalytic tyrosine and the 3'-end of the broken DNA. Tyrosyl-DNA phosphodiesterase (Tdp1) can repair Top1-DNA covalent complexes by hydrolyzing the tyrosyl-DNA bond. Inhibiting Tdp1 has the potential to enhance the anticancer activity of Top1 inhibitors (<http://discover.nci.nih.gov/pommier/pommier.htm>) and to act as antiproliferative agents. In the present study, we report that neomycin inhibits

Tdp1 more effectively than the related aminoglycosides paromomycin and lividomycin A. Inhibition of Tdp1 by neomycin is observed both with single- and double-stranded substrates but is slightly stronger with duplex DNA, which is different from aclarubicin, which only inhibits Tdp1 with the double-stranded substrate. Inhibition by neomycin can be overcome with excess Tdp1 and is greatest at low pH. To our knowledge, aminoglycoside antibiotics and the ribosome inhibitors thiostrepton, clindamycin-2-phosphate, and puromycin are the first reported pharmacological Tdp1 inhibitors.

DNA topoisomerase I (Top1) is ubiquitous and essential in higher eukaryotes. It relieves DNA torsional stress and relaxes DNA supercoiling by introducing DNA single-strand breaks, which are produced by the covalent linking of the Top1 catalytic tyrosine residue (Y723 in humans) to a 3'-DNA phosphate. Thus, these breaks are referred to as "Top1 cleavage complexes" (Champoux, 2001; Wang, 2002). Once the DNA is relaxed, each break is religated as the 5'-end of the broken DNA reseals the break by attacking the phosphotyrosyl bond, which releases Top1. Top1-DNA cleavage complexes are normally undetectable because they are very transient (for review, see Pommier et al., 1998; Champoux, 2001; Wang, 2002).

Top1 cleavage complexes can selectively be trapped by the natural alkaloid camptothecin (Hsiang et al., 1985) as the drug binds at the enzyme-DNA interface and prevents DNA religation (Pommier and Cherfils, 2005). Two camptothecin derivatives are used in cancer therapy [topotecan (Hycamtin; GlaxoSmithKline, Uxbridge, Middlesex, UK) and irinotecan (CPT-11, Camptosar; Pfizer, New York, NY)], and several

families of noncamptothecin inhibitors are being developed as novel anticancer agents (Meng et al., 2003). Top1 cleavage complexes can also be trapped by endogenous DNA lesions, including abasic sites, mismatches, oxidized bases, nicks, and carcinogenic DNA adducts (Pourquier and Pommier, 2001; Pommier et al., 2003, 2006). Hence, DNA modifications such as those associated with oxidative damage (thousands per cell per day) can stabilize Top1 cleavage complexes (Pourquier and Pommier, 2001; Sordet et al., 2004). In contrast to camptothecins and other Top1 inhibitory drugs, these DNA modifications produce irreversible cleavage complexes when the 5'-end of the DNA is irreversibly misaligned, as in the case of abasic sites or DNA breaks (Pourquier and Pommier, 2001; Pommier et al., 2003, 2006). The irreversible cleavage complexes are commonly referred to as "suicide complexes". Reversible cleavage complexes trapped by drugs can also be converted into irreversible complexes after collision of replication forks or transcription complexes with the Top1 cleavage complexes (reviewed in Pommier et al., 2003).

Tyrosyl-DNA phosphodiesterase (Tdp1) was discovered as an enzyme that specifically removes the 3'-phosphotyrosyl adducts (Yang et al., 1996; Pouliot et al., 1999). Top1 needs to be proteolyzed (Debethune et al., 2002) or denatured (Interthal et al., 2005a) for Tdp1 to hydrolyze the tyrosyl-DNA bond. Top1 degradation and ubiquitination have indeed been observed after camptothecin treatment (Desai et al., 1997).

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ABBREVIATIONS: Top1, DNA topoisomerase I; Tdp1, human tyrosyl-DNA phosphodiesterase; PLD, phospholipase D; SCAN1, spinocerebellar ataxia with axonal neuropathy; Neo, neomycin; PAGE, polyacrylamide gel electrophoresis.

Tdp1 orthologs are present in all eukaryotic species examined, including yeasts and humans (Pouliot et al., 1999; Interthal et al., 2001). Sequence comparisons (Interthal et al., 2001) and structural studies (Davies et al., 2002b) revealed that Tdp1 is a member of the phospholipase D (PLD) superfamily, which also includes a bacterial toxin, poxvirus envelope proteins, and bacterial nucleases (Interthal et al., 2001).

In humans, homozygous mutation in the *TDP1* gene (1478A-G) resulting in substitution of histine 493 with arginine is responsible for "spinocerebellar ataxia with axonal neuropathy" (SCAN1) (Takashima et al., 2002; Paulson and Miller, 2005). Recent studies demonstrated that SCAN1 cells are hypersensitive to camptothecin (Interthal et al., 2005b) and that Tdp1 is required for the repair of abortive Top1 cleavage complexes (El-Khamisy et al., 2005). Tdp1 forms multiprotein complexes with the single-strand break repair XRCC1 complexes (Plo et al., 2003) by direct interaction with DNA ligase III (El-Khamisy et al., 2005). These complexes are catalytically defective in SCAN1 cell extracts (El-Khamisy et al., 2005), which accumulate Tdp1-DNA intermediates (Interthal et al., 2005b). Tdp1 can also remove glycolate residues from the 3'-end of DNA (Inamdar et al., 2002). 3'-Phosphoglycolate is a common byproduct of DNA double-strand breaks caused by oxidative fragmentation of DNA sugars, which occur as a result of ionizing radiation and oxidative DNA damage (Inamdar et al., 2002). Consistently, extracts from SCAN1 cells are deficient in processing 3'-phosphoglycolate (Zhou et al., 2005). Thus, Tdp1 seems to repair Top1-DNA adducts and free-radical-mediated DNA breaks. Because the latter can also generate Top1 covalent complexes (Pourquier and Pommier, 2001), Top1 repair is probably a critical function of Tdp1.

In budding yeast, a T722A mutant Top1 that induces high level of cleavage complexes by increasing their stability results in low viability (Pouliot et al., 2001). However, Tdp1 deficiency alone does not confer hypersensitivity to Top1 cleavage complexes unless an additional mutation of the *RAD9* checkpoint gene (Pouliot et al., 2001) or the *RAD1* endonuclease gene (Liu et al., 2002; Vance and Wilson, 2002) is associated with a TDP1-null mutation (reviewed in Pommier et al., 2003, 2006 and at <http://discover.nci.nih.gov/pommier/pommier.htm>). Moreover, Tdp1 overexpression in human cells counteracts DNA damage mediated not only by Top1 but also by Top2 (Barthelmes et al., 2004). Because cancer cells are characteristically defective in checkpoint and DNA repair, and oncogenic transformation produces high levels of oxidative radicals, it is plausible that Tdp1 inhibitors might be used for anticancer treatment alone or more likely in combination with camptothecins or other Top1 inhibitors.

The present study reports the first pharmacological inhibitors for Tdp1. The only other inhibitors of Tdp1 are vanadate and tungstate, which are general inhibitors of a variety of enzymes involved in phosphoryl transfer reactions (Davies et al., 2002a). Using recombinant human Tdp1 and model tyrosyl-oligonucleotides substrates, we show that antibiotics that target bacterial ribosomes can inhibit Tdp1 activity.

Materials and Methods

Drugs and Reagents. Neomycin (Neo) and other chemicals were from Sigma-Aldrich (St. Louis, MO). High-performance liquid chro-

matography-purified oligonucleotides were purchased from the Midland Certified Reagent Co. (Midland, TX).

Preparation of Human Tdp1. Human Tdp1 expressing plasmid pHN1910 (a gift from Dr. Howard Nash, Laboratory of Molecular Biology, National Institute of Mental Health, National Institutes of Health) was constructed using vector pET-15b (Novagen, Madison, WI) with full-length human Tdp1 and an additional His-tag sequence of MGSSHHHHHHSSGLVPRGSHMLEDP in its N terminus. The His-tagged human Tdp1 was purified from Novagen BL21 cells using HiTrap Chelating HP (GE Healthcare, Little Chalfont, Buckinghamshire, UK) according to the company's protocol. Samples were assayed immediately. Tdp1 fractions were pooled and dialyzed with dialysis buffer (10% glycerol, 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 10 mM β -mercaptoethanol, and 2 mM EDTA). Dialyzed samples were aliquoted and stored at -80°C . Tdp1 concentration was determined using Bradford protein assay (Bio-Rad Laboratories, Hercules, CA), and its purity was analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (see Fig. 1B).

Preparation of Tdp1 Substrates. High-performance liquid chromatography-purified oligonucleotides N14Y (see Fig. 1A) (Plo et al., 2003) were labeled at their 5'-end with [γ - ^{32}P]ATP (PerkinElmer Life and Analytical Sciences, Boston, MA) by incubation with 3'-phosphatase-free T4 polynucleotide kinase (Roche Applied Science, Indianapolis, IN) according to the manufacturer's protocols. Unincorporated nucleotides were removed by Sephadex G-25 spin-column chromatography (Mini Quick Spin Oligo columns; Roche Applied Science). For the production of the oligonucleotide duplexes D14Y, N14Y was mixed with the complementary oligonucleotide (see Fig. 1A) in equal molar ratios in annealing buffer (10 mM Tris-HCl, pH

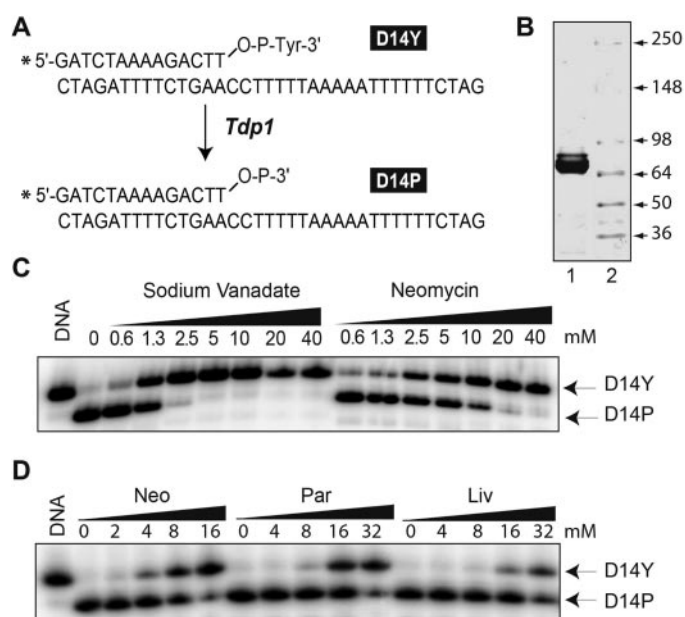


Fig. 1. Inhibition of Tdp1 activity by neomycin B (Neo), paromomycin I (Par), and lividomycin (Liv). A, schematic representation of the Tdp1 biochemical assay. The partially duplex oligopeptide D14Y was used as a substrate (Debethune et al., 2002; Plo et al., 2003). ^{32}P -Radiolabeling (*) was at the 5' terminus of the upper 14mer strand. Tdp1 catalyzes the hydrolysis of the 3'-phosphotyrosine bond and converts D14Y to an oligonucleotide with 3'-phosphate (D14P). B, representative SDS-PAGE gel showing the purified recombinant Tdp1 used in this study (Coomassie blue staining; lane 1). Lane 2 shows markers with molecular mass (in kilodaltons) at right. C, representative gel showing Tdp1 inhibition by vanadate and neomycin. D, representative gel showing Tdp1 inhibition by neomycin, paromomycin, and lividomycin. Reactions were performed at pH 8.0 with 25 nM D14Y, 1 ng of Tdp1, and the indicated inhibitors at 25°C for 20 min. Drug concentrations are indicated above gel picture. The 3'-phosphate oligonucleotide product (D14P) runs more quickly than the corresponding tyrosyl oligonucleotide substrate (D14Y) in PAGE.

7.5, 100 mM NaCl, and 10 mM MgCl_2), heated to 96°C, and allowed to cool down slowly (over 2 h) to room temperature.

Tdp1 Assays. Unless indicated otherwise, Tdp1 assays were performed in 20- μl mixtures containing 50 mM Tris-HCl, pH 8.0, 80 mM KCl, 2 mM EDTA, 1 mM dithiothreitol, and 40 $\mu\text{g}/\text{ml}$ bovine serum albumin. For initial screening of Tdp1 inhibitors, 25 nM 5'- ^{32}P -labeled substrate (D14Y) was reacted with 1 ng of Tdp1 (≈ 0.7 nM) in the absence or presence of inhibitor for 20 min at 25°C. Reactions were stopped by the addition of 60 μl of gel loading buffer [98% (v/v) formamide, 1% (w/v) xylene cyanol, and 1% (w/v) bromophenol blue]. Twelve-microliter aliquots were resolved in 20% denaturing polyacrylamide (AccuGel; National Diagnostics, Atlanta, GA) (19:1) gel containing 7 M urea. After drying, gels were exposed overnight to PhosphorImager screens (GE Healthcare). Screens were scanned, and images were obtained with the Molecular Dynamics software. Densitometry analyses were performed using ImageQuant 5.2 software package (GE Healthcare). Tdp1 activity was determined by measuring the fraction of substrate converted into 3'-phosphate DNA product by densitometry analysis of the gel image (Debethune et al., 2002). Figures show representative results that were consistently reproduced at least three times.

Results

Inhibition of Tdp1 by Aminoglycosides and Other Antibiotic Ribosome Inhibitors. It was reported that sodium vanadate, a phosphatase inhibitor, inhibits Tdp1 activity (Davies et al., 2002a). As expected, vanadate inhibited Tdp1 under our assay conditions (Fig. 1C). Its inhibitory activity has been related to its phosphate-mimicking activity

and/or its activity as a transition-state analog (Davies et al., 2002a, 2003, 2004). Because Tdp1 is a member of the phospholipase D superfamily (Davies et al., 2002b), and neomycin was reported to inhibit PLD (Huang et al., 1999), we tested whether neomycin could also inhibit Tdp1 activity. Using Tdp1 biochemical assays (Fig. 1A), we found that neomycin also inhibits purified recombinant Tdp1 (Fig. 1, C and D). The two neomycin analogs, paromomycin and lividomycin, also inhibited Tdp1 activity at slightly higher concentrations (Fig. 1D). Because neomycin, paromomycin, lividomycin, and other aminoglycosides are known inhibitors of bacterial ribosomes (Schroeder et al., 2000), we tested other aminoglycosides and nonaminoglycoside ribosomal inhibitors (Moore and Steitz, 2003). Figure 2A shows the structures and Tdp1 inhibitors activity of various ribosomal inhibitors against Tdp1. The most active inhibitors were neomycin (IC_{50} , 8 mM) and thiostrepton (1.8 mM). However, some activity was found for clindamycin-2-phosphate and puromycin, albeit at higher concentrations. Although these concentrations are high, reactions were performed at saturating Tdp1 activity (20-min reactions with 1 ng of Tdp1; see Figs. 4A and 5 for greater inhibition under different conditions).

Neomycin Inhibits the Processing of Both Duplex and Single-Stranded DNA by Tdp1. As partially duplex DNA and single-stranded DNA are both substrates for Tdp1 (Yang et al., 1996; Pouliot et al., 2001; Davies et al., 2003), we compared the inhibition of Tdp1 by neomycin using the D14Y

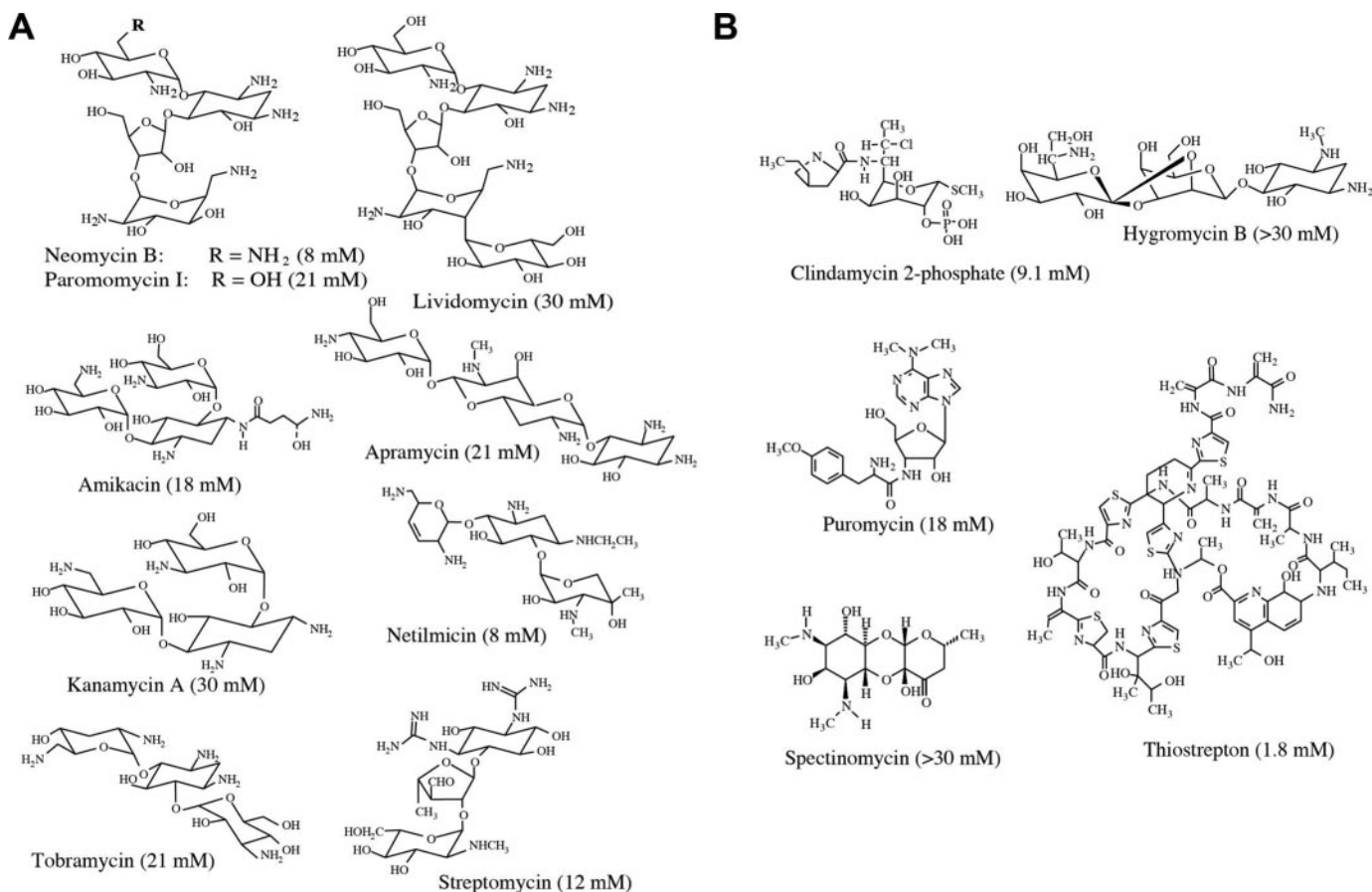


Fig. 2. Structure-activity of aminoglycoside (A) and nonaminoglycoside (B) antibiotics. Drug concentrations required to inhibit 50% Tdp1 activity (IC_{50}) were derived from dose-response curves in reactions performed for 20 min at pH 8.0 and 25°C in the presence of 25 nM D14Y substrate and 1 ng of Tdp1 (see Fig. 1).

and N14Y substrates (Fig. 3). Figure 3, A and B, shows that neomycin inhibits the processing of both the single- and double-stranded substrates by Tdp1, although neomycin was slightly more effective with the duplex substrate. By contrast, sodium vanadate was similarly active with both single- and double-stranded substrates, and aclarubicin, a known DNA intercalator, inhibited Tdp1 selectively with double-stranded DNA (Fig. 3C). We conclude that neomycin inhibits Tdp1 activity both with single- and double-stranded DNA substrates.

Kinetics of Tdp1 Inhibition by Neomycin. As mentioned above, the initial assays (Figs. 1–3) had been performed at one time point (20 min) under conditions where Tdp1 fully converts the substrate in the absence of inhibitor (1 ng, pH 8.0). Figure 4 (A, left; and circles in B and C) shows that under these conditions, Tdp1 converted more than 90% of the D14Y substrate within 3 min. Thus, we wished to determine whether concentrations of neomycin below its determined IC_{50} would affect the kinetics of Tdp1 activity. Tdp1 activity was slowed down at 1 mM neomycin, a concentration producing no detectable inhibition after a 20-min reaction (Fig. 4, A and B). Kinetic plots demonstrated that 1 mM neomycin increased the conversion half-time ($t_{1/2}$) of D14Y from 0.5 min in the absence of drug to 3 min in the presence of 1 mM neomycin and 8 min in the presence of 2 mM neomycin (Fig. 4C). These experiments suggest that neomycin produces reversible inhibition of Tdp1.

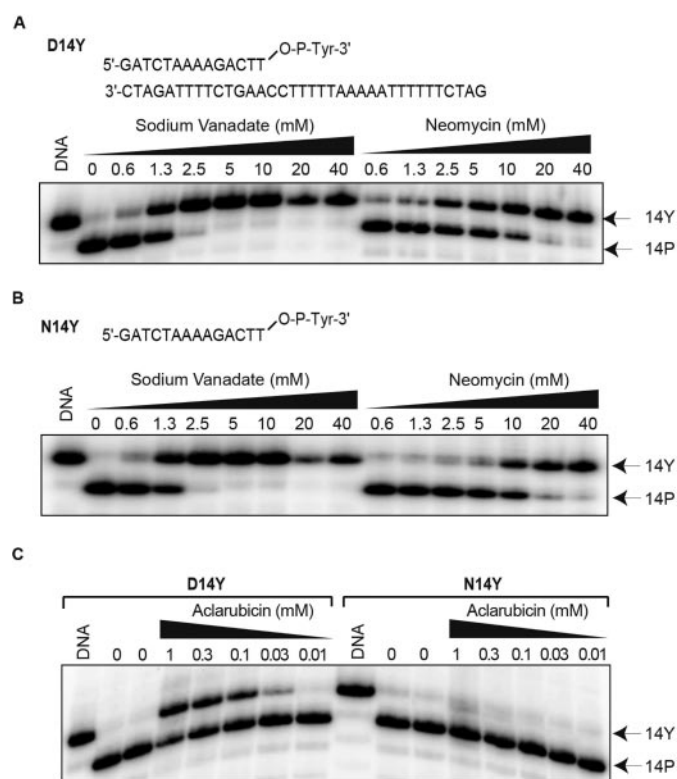


Fig. 3. Neomycin inhibits Tdp1 activity both with duplex and single-stranded substrates but is more effective with the duplex substrate. A, inhibition of Tdp1 by neomycin and sodium vanadate using the partially duplex substrate (D14Y; sequence shown at the top). B, inhibition of Tdp1 by neomycin and sodium vanadate using the single-stranded substrate (N14Y; sequence shown at the top). C, inhibition of Tdp1 by the intercalator aclarubicin using the partially duplex substrate (D14Y). No Tdp1 inhibition was observed in the presence of aclarubicin using the single-stranded N14Y substrates.

Tdp1 Inhibition by Neomycin Depends on Time of Addition, Tdp1 Concentration, and Reaction pH. To test whether DNA binding contributes to the inhibitory effect of neomycin, we performed order-of-addition experiments. Preincubating the DNA substrate for 20 min with neomycin before the addition of Tdp1 diminished the inhibitory potency of neomycin (compare open and closed triangles in Fig. 4C). By contrast, preincubating neomycin with Tdp1 for 20 min before adding the DNA gave inhibition similar to that shown by experiments done without preincubation (data not shown). These results suggest that Tdp1 is preferentially inhibited when neomycin binds to the Tdp1-DNA complex (see *Discussion*).

Increasing Tdp1 concentration was able to overcome Tdp1 inhibition by neomycin even when neomycin was present at 10 mM drug concentration (Fig. 5, A and B). Thus, free Tdp1 competes with neomycin. Moreover, if DNA were the only target of neomycin, inhibition should not depend on Tdp1 concentration, which is not what we observed (Fig. 5, A and B). These results suggest that neomycin inhibits Tdp1 by binding directly to Tdp1.

Neomycin tends to be protonated and to bind nucleic acids better at acidic pH than at pH 8.0, which is the pH used in the previous experiments and in previous publications (Pouliot et al., 1999, 2001; Debethune et al., 2002; Raymond et al., 2004). Because nuclear pH is also acidic, we examined Tdp1 inhibition by neomycin at pH 6.4. Figure 5C shows that Tdp1 activity decreases with pH (Raymond et al., 2005). Nevertheless, after a 20-min reaction (dotted line in Fig. 5C), substrate processing was nearly the same at pH 6.4 and 8. Under these conditions, neomycin was more effective at pH 6.4 than at pH 8.0 (IC_{50} , 1.8 versus 8 mM, respectively) (Fig. 5D).

To gain an appreciation of neomycin IC_{50} at physiological pH under nonsaturating conditions, we lowered Tdp1 concentration to 0.1 ng and the Tdp1 reaction time to 8 min. Figure 5D shows that under these conditions, the IC_{50} for neomycin was approximately 0.25 mM, which is in the range of concentrations for inhibition of the ribosome and the spliceosome.

Discussion

This study suggests that antibiotics could serve as a basis for the discovery and design of Tdp1 inhibitors. Tdp1 is conserved from yeast to humans, which indicates its functional importance. Tdp1 is involved in the removal of covalent Top1-DNA complexes after degradation or denaturation of the Top1 polypeptide covalently linked to the 3'-end of DNA (Yang et al., 1996; Debethune et al., 2002; Interthal et al., 2005a). Tdp1 is also involved in the repair of oxidative damage by removing glycolate residues from the 3'-end of DNA breaks (Inamdar et al., 2002; Zhou et al., 2005). The development of Tdp1 inhibitors as anticancer agents can be envisioned as combinations of Tdp1 and Top1 inhibitors. Tumor cells, whose repair pathways are commonly deficient, might be selectively sensitized to Top1 inhibitors compared with normal cells that contain redundant repair pathways. Moreover, Tdp1 inhibitors might also be effective by themselves as anticancer agents because oncogenic activation tends to increase free radical production and genomic instability (Cerutti, 1985). In addition, Tdp1 inhibitors might be valuable as anti-infectious agents because the gene is present in parasites.

The inhibitors reported in the present study all bind RNA motifs present in bacterial ribosomes. Neomycin is a polycationic aminoglycoside antibiotic with a four-membered ring structure consisting of a 2-deoxystreptamine ring linked to several amino sugars (Kotra et al., 2000) (see Fig. 2A). Neomycin interacts with the 16S rRNA of the 30S ribosomal subunits within an internal loop in the decoding site (Schroeder et al., 2000). Binding between the rRNA of the internal loop and rings I and II of the aminoglycoside antibiotic distort the A-site and leads to amino acid misincorporation (Schroeder et al., 2000; Ogle et al., 2001; Moore and Steitz, 2003). Neomycin, which differs in structure from paromomycin by the change of single amino group by hydroxyl on

the C'-6 position of ring I (Fig. 2A), showed approximately 2-fold greater inhibition for Tdp1 than paromomycin and lividomycin (Fig. 2A). Protonation of the neomycin amino groups is probably important for inhibition because neomycin was more effective at pH 6.4 than pH 8.0 (approximately 4-fold) (Fig. 5D). The same increase in activity at acidic pH (pH 6.4 is close to cellular nuclear pH) was also observed for paromomycin and lividomycin (data not shown), which indicates that protonation increases the inhibitory activity of aminoglycosides.

Although protonation increases the binding of neomycin to RNA, our experiments suggest that the inhibitory effect of neomycin is probably not simply related to nucleic acid bind-

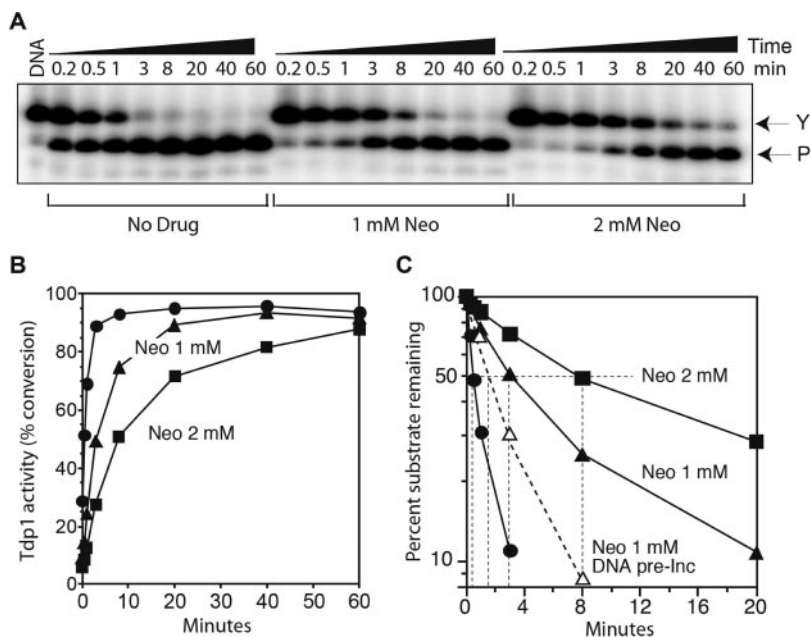


Fig. 4. Kinetics of Tdp1 inhibition by neomycin. A, a 100- μ l reaction mixture containing 25 nM D14Y and 5 ng of Tdp1 was incubated at pH 8.0 at 25°C in the absence of drug (No Drug), or in the presence of 1 or 2 mM Neo. Aliquots were taken at the indicated times. Reaction products were analyzed by denaturing PAGE. Substrate D14Y (Y) and product D14P (P) are shown in a representative gel. B, densitometry analysis of the gel shown in A. Tdp1 activity was calculated as the percentage of D14Y converted to D14P. \bullet , no drug; \blacktriangle , 1 mM Neo; and \blacksquare , 2 mM Neo. C, kinetics and half-lives ($T_{1/2}$, vertical dotted lines) for Tdp1 activity measured as the percentage of DNA substrate (D14Y) remaining as a function of incubation time. In the absence of drug (\bullet), $T_{1/2} \approx 0.5$ min; in the presence of 1 mM Neo (\blacktriangle) $T_{1/2} \approx 3$ min; and in the presence of 2 mM Neo (\blacksquare), $T_{1/2} \approx 8$ min. Reaction were also performed by preincubating the DNA substrate (D14Y) for 20 min before the addition of Tdp1 for an additional 20 min (\triangle).

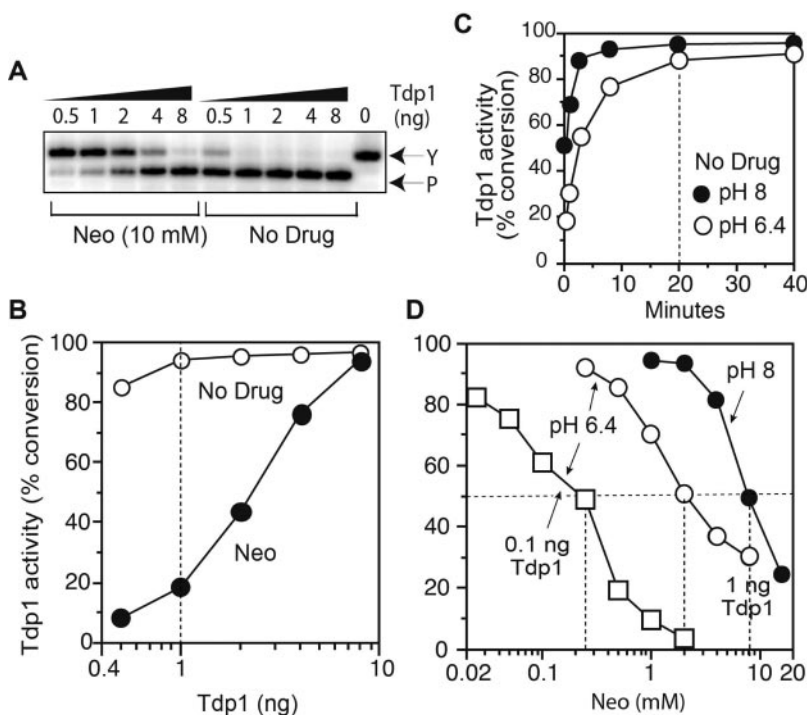


Fig. 5. Inhibition of Tdp1 by neomycin is dependent on both Tdp1 concentration and pH. A, 20- μ l reaction mixtures contained 25 nM D14Y and increasing amounts of Tdp1 (0.5–8 ng) in the absence or presence of 10 mM neomycin. Reactions were at 25°C, pH 8, for 20 min. A representative gel is shown. B, graphical representation of Tdp1 inhibition by neomycin (\bullet). Tdp1 activity was calculated as the percentage of D14Y converted to D14P. The vertical dotted line corresponds to the Tdp1 concentration used in Figs. 1 to 4. C, Tdp1 activity is pH-dependent. Reactions were performed at the indicated pH with 25 nM D14Y and 1 ng of Tdp1 at 25°C for 20 min. D, Tdp1 inhibition by neomycin depends both on pH (pH 8.0, \bullet ; pH 6.4, open symbols) and Tdp1 concentration (1 ng of Tdp, circles; 0.1 ng, squares). Reactions were for 8 min, and Tdp1 activity was calculated as the percentage of D14Y converted to D14P.

ing. We found no Tdp1 inhibition for the polyamines spermine and spermidine (our unpublished data). If neomycin was primarily targeting the DNA substrate, one would have expected that preincubation of neomycin with DNA would enhance inhibition. However, preincubation experiments (Fig. 4C) showed that neomycin was less efficient against Tdp1 when it was first incubated with DNA. In our experiments, the tyrosyl-DNA substrate is at much lower concentration than neomycin. The concentration ratio is $25:10^6$ (i.e., the drug is in 40,000-fold excess compared with the DNA). Therefore, it is unlikely that binding of neomycin to the DNA substrate would reduce the concentration of free drug. Moreover, if DNA were the main target of neomycin, increasing Tdp1 concentration would not have been expected to affect Tdp1 inhibition by neomycin, which is not the case because inhibition is in fact inversely related to the Tdp1 concentration (Fig. 5, A and B). In addition, neomycin inhibits Tdp1 activity toward both single- and double-stranded DNA substrates (Fig. 3), whereas neomycin is known to bind duplex RNA (Schroeder et al., 2000; Moore and Steitz, 2003). Therefore, we propose that neomycin binds either directly to Tdp1 or to the Tdp1-DNA complex interface. The latter possibility would characterize neomycin as a potential interfacial inhibitor. However, if neomycin stabilizes the Tdp1-DNA intermediate, we were not able to detect the resulting complex at the top of the gels under the electrophoresis conditions (Interthal et al., 2005b). Interfacial inhibition has recently emerged as a common mechanism for natural drugs against a variety of targets including protein-DNA interfaces in the case of camptothecin and antibiotics (Pommier and Marchand, 2005) and protein-protein interfaces in the case brefeldin A, colchicine, paclitaxel, or vinblastine as tubulin inhibitors (Pommier and Cherfils, 2005).

Further studies are warranted to determine the Tdp1 binding site of aminoglycoside antibiotics, clindamycin-2-phosphate, puromycin, and thiostrepton (see Fig. 2). Neomycin is also known to inhibit (bind to) phospholipase D, which does not contain RNA or DNA (Huang et al., 1999). Neomycin acts as an uncompetitive inhibitor of PLD by binding to the PLD activator phosphatidylinositol 4,5-bisphosphate (PIP2) or, alternatively, to the PIP2-PLD complex to form a ternary complex (Huang et al., 1999).

Several crystal structures of human Tdp1 have been determined in the absence or presence of peptide-nucleic acid ligands (Interthal et al., 2001; Davies et al., 2002a,b, 2003, 2004). Although Tdp1 contains a positively charged groove that accommodates the nucleic acid substrate, it also contains clusters of negatively charged residues in the vicinity of the active site (Davies et al., 2003). These acidic residues might bind the neomycin polycations. Cocrystallization of the antibiotics described is warranted to elucidate the drug binding site and the potential contribution of the peptide-nucleic acid substrate for drug binding to Tdp1. Such studies may also guide the discovery and design of more potent and more selective Tdp1 inhibitors.

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References

- Barthelme HU, Habermeyer M, Christensen MO, Mielke C, Interthal H, Pouliot JJ, Boege F, and Marko D (2004) TDP1 overexpression in human cells counteracts DNA damage mediated by topoisomerases I and II. *J Biol Chem* **279**:55618–55625.
- Cerutti PA (1985) Prooxidant states and tumor promotion. *Science (Wash DC)* **227**:375–381.
- Champoux JJ (2001) DNA topoisomerases: structure, function and mechanism. *Annu Rev Biochem* **70**:369–413.
- Davies DR, Interthal H, Champoux JJ, and Hol WG (2002a) Insights into substrate binding and catalytic mechanism of human tyrosyl-DNA phosphodiesterase (Tdp1) from vanadate and tungstate-inhibited structures. *J Mol Biol* **324**:917–932.
- Davies DR, Interthal H, Champoux JJ, and Hol WG (2002b) The crystal structure of human tyrosyl-DNA phosphodiesterase, Tdp1. *Structure (Camb)* **10**:237–248.
- Davies DR, Interthal H, Champoux JJ, and Hol WG (2003) Crystal structure of a transition state mimic for tdp1 assembled from vanadate, DNA and a topoisomerase I-derived peptide. *Chem Biol* **10**:139–147.
- Davies DR, Interthal H, Champoux JJ, and Hol WG (2004) Explorations of peptide and oligonucleotide binding sites of tyrosyl-DNA phosphodiesterase using vanadate complexes. *J Med Chem* **47**:829–837.
- Debethune L, Kohlhaagen G, Grandas A, and Pommier Y (2002) Processing of nucleopeptides mimicking the topoisomerase I-DNA covalent complex by tyrosyl-DNA phosphodiesterase. *Nucleic Acids Res* **30**:1198–1204.
- Desai SD, Liu LF, Vazquez-Abad D, and D'Arpa P (1997) Ubiquitin-dependent destruction of topoisomerase I is stimulated by the antitumor drug camptothecin. *J Biol Chem* **272**:24159–24164.
- El-Khamisy SF, Saifi GM, Weinfeld M, Johansson F, Helleday T, Lupski JR, and Caldecott KW (2005) Defective DNA single-strand break repair in spinocerebellar ataxia with axonal neuropathy-1. *Nature (Lond)* **434**:108–113.
- Hsiang YH, Hertzberg R, Hecht S, and Liu LF (1985) Camptothecin induces protein-linked DNA breaks via mammalian DNA topoisomerase I. *J Biol Chem* **260**:14873–14878.
- Huang Y, Qureshi IA, and Chen H (1999) Effects of phosphatidylinositol 4,5-bisphosphate and neomycin on phospholipase D: kinetic studies. *Mol Cell Biochem* **197**:195–201.
- Inamdar KV, Pouliot JJ, Zhou T, Lees-Miller SP, Rasouli-Nia A, and Povirk LF (2002) Conversion of phosphoglycolate to phosphate termini on 3' overhangs of DNA double-strand breaks by the human tyrosyl-DNA phosphodiesterase hTdp1. *J Biol Chem* **277**:27162–27168.
- Interthal H, Chen HJ, and Champoux JJ (2005a) Human Tdp1 cleaves a broad spectrum of substrates including phosphoamide linkages. *J Biol Chem* **280**:36518–36528.
- Interthal H, Chen HJ, Kehl-Fie TE, Zotzmann J, Leppard JB, and Champoux JJ (2005b) SCAN1 mutant Tdp1 accumulates the enzyme-DNA intermediate and causes camptothecin hypersensitivity. *EMBO (Eur Mol Biol Organ) J* **24**:2224–2233.
- Interthal H, Pouliot JJ, and Champoux JJ (2001) The tyrosyl-DNA phosphodiesterase Tdp1 is a member of the phospholipase D superfamily. *Proc Natl Acad Sci USA* **98**:12009–12014.
- Kotra LP, Haddad J, and Mobashery S (2000) Aminoglycosides: perspectives on mechanisms of action and resistance and strategies to counter resistance. *Antimicrob Agents Chemother* **44**:3249–3256.
- Liu C, Pouliot JJ, and Nash HA (2002) Repair of topoisomerase I covalent complexes in the absence of the tyrosyl-DNA phosphodiesterase Tdp1. *Proc Natl Acad Sci USA* **99**:14970–14975.
- Meng L-H, Liao Z-Y, and Pommier Y (2003) Non-camptothecin DNA topoisomerase I inhibitors in cancer chemotherapy. *Curr Topics Med Chem* **3**:305–320.
- Moore PB and Steitz TA (2003) The structural basis of large ribosomal subunit function. *Annu Rev Biochem* **72**:813–850.
- Ogle JM, Brodersen DE, Clemons WM Jr, Tarry MJ, Carter AP, and Ramakrishnan V (2001) Recognition of cognate transfer RNA by the 30S ribosomal subunit. *Science (Wash DC)* **292**:897–902.
- Paulson HL and Miller VM (2005) Breaks in coordination: DNA repair in inherited ataxia. *Neuron* **46**:845–848.
- Plo I, Liao ZY, Barcelo JM, Kohlhaagen G, Caldecott KW, Weinfeld M, and Pommier Y (2003) Association of XRCC1 and tyrosyl DNA phosphodiesterase (Tdp1) for the repair of topoisomerase I-mediated DNA lesions. *DNA Repair (Amst)* **2**:1087–1100.
- Pommier Y, Barcelo J, Rao VA, Sordet O, Jobson AG, Thibaut L, Miao Z, Seiler J, Zhang H, Marchand C, et al. (2006) Repair of topoisomerase I-mediated DNA damage. *Prog Nucleic Acid Res Mol Biol*, in press.
- Pommier Y and Cherfils J (2005) Interfacial protein inhibition: a nature's paradigm for drug discovery. *Trends Pharmacol Sci* **28**:136–145.
- Pommier Y and Marchand C (2005) Interfacial inhibitors of protein-nucleic acid interactions. *Curr Med Chem Anti-Cancer Agents* **5**:421–429.
- Pommier Y, Pourquier P, Fan Y, and Strumberg D (1998) Mechanism of action of eukaryotic DNA topoisomerase I and drugs targeted to the enzyme. *Biochim Biophys Acta* **1400**:83–105.
- Pommier Y, Redon C, Rao VA, Seiler JA, Sordet O, Takemura H, Antony S, Meng L, Liao Z, Kohlhaagen G, et al. (2003) Repair of and checkpoint response to topoisomerase I-mediated DNA damage. *Mutat Res* **532**:173–203.
- Pouliot JJ, Robertson CA, and Nash HA (2001) Pathways for repair of topoisomerase I covalent complexes in *Saccharomyces cerevisiae*. *Genes Cells* **6**:677–687.
- Pouliot JJ, Yao KC, Robertson CA, and Nash HA (1999) Yeast gene for a Tyr-DNA phosphodiesterase that repairs topoisomerase I covalent complexes. *Science (Wash DC)* **286**:552–555.
- Pourquier P and Pommier Y (2001) Topoisomerase I-mediated DNA damage. *Adv Cancer Res* **80**:189–216.
- Raymond AC, Rideout MC, Staker B, Hjerrild K, and Burgin AB Jr (2004) Analysis of human tyrosyl-DNA phosphodiesterase I catalytic residues. *J Mol Biol* **338**:895–906.

- Raymond AC, Staker BL, and Burgin AB Jr (2005) Substrate specificity of tyrosyl-DNA phosphodiesterase I (Tdp1). *J Biol Chem* **280**:22029–22035.
- Schroeder R, Waldsich C, and Wank H (2000) Modulation of RNA function by aminoglycoside antibiotics. *EMBO (Eur Mol Biol Organ) J* **19**:1–9.
- Sordet O, Khan QA, and Pommier Y (2004) Apoptotic topoisomerase I-DNA complexes induced by oxygen radicals and mitochondrial dysfunction. *Cell Cycle* **3**:1095–1097.
- Takashima H, Boerkoel CF, John J, Saifi GM, Salih MA, Armstrong D, Mao Y, Quijcho FA, Roa BB, Nakagawa M, et al. (2002) Mutation of TDP1, encoding a topoisomerase I-dependent DNA damage repair enzyme, in spinocerebellar ataxia with axonal neuropathy. *Nat Genet* **32**:267–272.
- Vance JR and Wilson TE (2002) Yeast Tdp1 and Rad1-Rad10 function as redundant pathways for repairing Top1 replicative damage. *Proc Natl Acad Sci USA* **99**:13669–13674.
- Wang JC (2002) Cellular roles of DNA topoisomerases: a molecular perspective. *Nat Rev Mol Cell Biol* **3**:430–440.
- Yang S-W, Burgin AB, Huizenga BN, Robertson CA, Yao KC, and Nash HA (1996) A eukaryotic enzyme that can disjoin dead-end covalent complexes between DNA and type I topoisomerases. *Proc Natl Acad Sci USA* **93**:11534–11539.
- Zhou T, Lee JW, Tatavarthi H, Lupski JR, Valerie K, and Povirk LF (2005) Deficiency in 3'-phosphoglycolate processing in human cells with a hereditary mutation in tyrosyl-DNA phosphodiesterase (TDP1). *Nucleic Acids Res* **33**:289–297.

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