Salicylate, a catalytic inhibitor of topoisomerase II, inhibits DNA cleavage and is selective for the alpha isoform

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Abstract

Topoisomerase II (topo II) is a ubiquitous enzyme that is essential for cell survival through its role in regulating DNA topology and chromatid separation. Topo II can be poisoned by common chemotherapeutics (such as doxorubicin and etoposide), leading to the accumulation of cytotoxic enzyme-linked DNA double-stranded breaks. In contrast, non-break-inducing topo II catalytic inhibitors have also been described and have more limited use in clinical chemotherapy. These agents, however, may alter the efficacy of regimens incorporating topo II poisons. We previously identified salicylate, the primary metabolite of aspirin, as a novel catalytic inhibitor of topo II. We have now determined the mechanism by which salicylate inhibits topo II. As catalytic inhibitors can act at a number of steps in the topo II catalytic cycle, we used multiple independent, biochemical approaches to interrogate the catalytic cycle. Furthermore, as mammalian cells express two isoforms of topo II (α and β), we examined whether salicylate was isoform selective. Our results demonstrate that salicylate is unable to intercalate DNA, does not prevent enzyme-DNA interaction, nor does it promote stabilization of topo IIα in closed clamps on DNA. While salicylate decreased topo IIα ATPase activity in a dose-dependent non-competitive manner, this was secondary to salicylate-mediated inhibition of DNA cleavage. Surprisingly, comparison of salicylate’s effects using purified human topo IIα and topo IIβ revealed that salicylate selectively inhibits the alpha isoform. These findings provide a definitive mechanism for salicylate-mediated inhibition of topo IIα and provide support for further studies determining the basis for its isoform selectivity.
Introduction

For cells to package their genetic material within the restricted boundaries of a nucleus, DNA must be supercoiled and organized into discrete higher-order chromatin structures. Yet, relaxation of chromatin must be similarly regulated to permit orderly DNA replication and gene expression. To accomplish these tasks, cells express at least one type IIA DNA topoisomerase, an essential enzyme required for the maintenance and regulation of DNA topology (Nitiss, 2009a; Vos et al., 2011).

The role of eukaryotic topoisomerase II (topo II) in DNA replication and gene expression has been well established (Nitiss, 2009a; Vos et al., 2011). As the DNA helix is unwound, positive and negative supercoils are generated in front of and trailing the replication machinery, respectively. These topological structures can act as physical barriers to replication and transcription and must be resolved (Baxter et al., 2011; Fachinetti et al., 2010). In addition, newly synthesized sister chromatids are intertwined after replication, requiring decatenation by topoisomerase II prior to chromosome segregation during anaphase (Germe et al., 2009; Luo et al., 2009). Mammalian cells express two isoforms of topo II (topo IIα and topo IIβ). While the alpha isoform is predominant in actively replicating cells and its expression level rises and falls through the cell cycle, the beta isoform is expressed at constant levels throughout the cell cycle and has been found to be necessary for regulated transcription and neural development (Ju et al., 2006; Vávrová and Šimůnek, 2012).
Chemotherapeutics targeting topo II have been of great utility in clinical oncology. Widely used chemotherapeutics such as doxorubicin and etoposide, chosen initially for their potency at inducing cell death, were subsequently discovered to target topo II. These agents, termed topo II poisons, are characterized by their ability to stabilize the topo II-DNA cleavable complex leading to the accumulation of DNA double-stranded breaks in the cell. However, treatment with topo II poisons is associated with an increased risk of secondary malignancies and cardiotoxicity (Lipshultz et al., 2010; Vrooman et al., 2011). Recent work has uncovered that these events are mediated through topo IIβ (Cowell et al., 2012; Zhang et al., 2012).

In addition to the well-known topo II poisons, a diverse range of compounds have been identified that inhibit topo II activity without stabilizing DNA strand breaks; these are collectively termed catalytic inhibitors and inhibit topo II at any one of several stages of its catalytic cycle (Larsen et al., 2003; Pommier, 2013). For example, intercalators, by distorting the DNA helical structure, interfere with topo II-DNA binding (Braña et al., 2001; Sørensen et al., 1992), ATPase inhibitors prevent ATP hydrolysis and/or the release of ADP and free phosphate that is required for strand passage (Hu et al., 2006; Vaughn et al., 2005), and bisdioxopiperazines, including ICRF-187 and ICRF-193, inhibit the enzyme in a closed clamp conformation, such that the enzyme cannot be released from DNA after religation (Classen et al., 2003; Germe and Hyrien, 2005; Morris et al., 2000). These compounds have been examined for therapeutic potential, and have been shown to reduce both topo II poison-associated cardiotoxicity and the incidence of secondary malignancies (Lipshultz et al., 2010; Vrooman et al., 2011). However, their use as primary agents in the treatment of cancer has not been realized.
We recently identified salicylate, the primary active metabolite of aspirin (acetylsalicylic acid) and one of the most widely used drugs worldwide, as a novel catalytic inhibitor of topo II (Bau and Kurz, 2011). Using cultured human breast cancer cells, we demonstrated that a brief pretreatment with salicylate decreases doxorubicin or etoposide-induced DNA damage by preventing topo II-mediated DNA double-stranded break formation, thereby decreasing the cytotoxicity of topo II poisons (Bau and Kurz, 2011). Our previous work demonstrated that these salicylate-mediated effects are specific for topo II and are independent of other known functions of salicylate (Bau and Kurz, 2011). We now extend our work to demonstrate the mechanism of topo II catalytic inhibition by salicylate and describe the selectivity of salicylate for the alpha isoform of topo II.
Materials and Methods

Reagents
Sodium salicylate, novobiocin, ICRF-193 and amsacrine (mAMSA) were purchased from Sigma-Aldrich (Oakville, Canada). Hypericin was purchased from EMD Millipore (Billerica, MA). Stock solutions of hypericin, mAMSA and ICRF-193 were prepared in dimethylsulfoxide (DMSO), protected from light and stored in aliquots at -20°C. Novobiocin was prepared in distilled H₂O and stored at -20°C. Salicylate was prepared freshly in distilled H₂O prior to each use. All other chemicals and reagents were of the highest quality available, nuclease free and purchased from Sigma-Aldrich or EMD Chemicals (Gibbstown, NJ). Recombinant human topo IIα was purchased from Topogen (Port Orange, FL) and recombinant apyrase was purchased from New England Biolabs (Ipswich, MA). Recombinant human topo IIβ was prepared as previously described (Austin et al., 1995).

Cell culture and preparation of cell extracts
For experiments requiring nuclear extracts, logarithmically growing MCF-7 human breast cancer cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Sigma-Aldrich) supplemented with 10% fetal bovine serum (Sigma-Aldrich). Cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂. Cells were grown to 70-80% confluence and harvested, washed twice in ice-cold phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄) and resuspended in hypotonic lysis buffer (30 mM Tris pH 7.5, 3 mM MgCl₂, 10 mM KCl, 20% v/v glycerol, 1% v/v Triton X-100) containing protease.
inhibitors (1 mM phenylmethylsulfonylfluoride, 2 μg ml⁻¹ aprotinin, 2 μg ml⁻¹ leupeptin, 1 μg ml⁻¹ pepstatin A). The cell suspension was then incubated on ice for 10 min prior to centrifugation at 12,000g for 2 min at 4°C. The supernatant was discarded and the nuclear pellet was resuspended in 300 mM NaCl extraction buffer (30 mM Tris pH 7.5, 3 mM MgCl₂, 300 mM NaCl) containing protease inhibitors. Following an additional 30 min incubation on ice, the suspension was centrifuged at 12,000g for 2 min at 4°C. Protein quantitation in the supernatant fraction was determined using a detergent-compatible protein assay (Bio-Rad, Hercules, CA) prior to snap-freezing aliquots in liquid nitrogen and storage at -80°C.

**Ethidium bromide displacement assay**

The displacement of ethidium bromide from DNA was measured as previously described (Fortune and Osheroff, 1998; Graves, 2001). In brief, increasing concentrations of salicylate were added to samples containing 5 nM of pBluescript-KS(+) plasmid and 1 μM ethidium bromide in a fluorescence buffer (10 mM HEPES pH 7.9, 100 mM KCl, 5 mM MgCl₂, 0.1 mM EDTA pH 8.0). Fluorescence emission spectra (λ max 595 nm; excitation 510 nm) were obtained in replicates of eight for each drug concentration and compared with those obtained using mAMSA, a topo II poison that is known to intercalate DNA and displace ethidium bromide at high drug concentrations.

**Topoisomerase I-mediated DNA unwinding assay**

As an alternative measure to examine DNA intercalation, a topoisomerase I (topo I)-based assay was carried out according to manufacturer’s protocol. In brief, recombinant human topo I (4 units, Topogen) was incubated with 150 ng supercoiled pHOT1 plasmid (Topogen) in a reaction
buffer (10 mM Tris-HCl pH 7.9, 1 mM EDTA pH 8.0, 150 mM NaCl, 1 μg μl⁻¹ bovine serum albumin, 0.1 mM spermidine and 5% v/v glycerol) at 37°C for 30 min. Subsequently, salicylate or mAMSA was added to the reaction and incubated a further 30 min at 37°C. Reactions were stopped by the addition of SDS to a final concentration of 1% w/v and the topo I was digested by the addition of proteinase K (50 μg) and incubation for 1 h at 56°C. Samples were resolved on a 1% w/v agarose gel containing 0.2 μg ml⁻¹ chloroquine in 1x TPE buffer (36 mM Tris pH 7.7, 1 mM EDTA, 30 mM NaH₂PO₄) overnight at 10 V. DNA bands were visualized after staining with 1 μg ml⁻¹ ethidium bromide.

**Electrophoretic mobility shift assay**

The binding of topo II to DNA was evaluated using electrophoretic mobility shift assays (EMSA) as previously described (Kurz et al., 2000; Peebles et al., 2001) with the following modifications. Oligonucleotides containing a strong topo II binding site corresponding to residues 87-126 of the pBR322 plasmid were annealed and end-labeled with [α-³²P]-dCTP (PerkinElmer, Waltham, MA, USA). Nuclear extracts (4 μg) from MCF-7 cells were incubated with ~5-10 fmol of the radiolabeled binding site in reaction buffer (20 mM Tris, pH 7.6, 50 mM KCl, 1 mM EDTA, 2 mM MgCl₂, 10% v/v glycerol, 0.5 μg poly[dl:dC]) on ice for 30 min. Reaction products were resolved on a 4% acrylamide / 0.5X TBE gel, dried using a gel dryer and exposed to film. For competition assays, reactions were incubated with increasing molar concentrations of unlabeled topo II binding site or an unlabeled non-specific double-stranded oligonucleotide. For supershift assays, a polyclonal rabbit antiserum raised to a fragment of human topo IIα (amino acids 857-1448) (a kind gift from Dr. David Kroll, Nature Research Center, North Carolina) was incubated with the extract alone for 30 min on ice prior the addition
of the radiolabeled oligo and continued incubation for 30 min on ice. Control experiments contained preimmune serum from the same animal. For experiments measuring the effect of salicylate on topo II-DNA binding, nuclear extract, salicylate and buffer were first incubated for 10 min on ice prior to the addition of radiolabeled binding site and continued incubation for 30 min. In experiments where purified topo IIα (Topogen) was used, reaction conditions were adjusted to include 1.6 fmol each of radiolabeled binding site and protein (1:1 ratio). The reaction buffer did not contain poly[dI:dC], but was otherwise identical to that used for nuclear extracts as were the binding conditions. Reaction products were resolved on a 6% acrylamide / 0.5X TBE gel and processed as described above.

**Closed clamp assay**

Topo IIα closed clamp formation was assayed as previously described (Roca, 2001; Roca and Wang, 1992) with modifications (Vaughn et al., 2005). Reactions contained topo IIα (33 nM), 0.6 μg (3 pmol) pBluescript-KS(+) plasmid DNA in reaction buffer A (10 mM Tris pH 7.7, 50 mM NaCl, 50 mM KCl, 0.1 mM EDTA, 10mM MgCl₂, 0.1 μg μl⁻¹ bovine serum albumin) with either 0.5 mM ATP, 0.5 mM AMP-PNP, or 0.5 mM ATP with 10 mM salicylate. Samples were incubated at 37°C for 5 min prior to stopping the reaction by the addition of NaCl/EDTA to final concentrations of 200 mM and 10 mM, respectively. Samples were then incubated at room temperature on glass fiber filters (GF/C, Whatman, Piscataway, NJ) for 10 min prior to centrifugation at 50g for 2 min. Filters were subsequently washed twice with buffer A, pooling the washes, followed by washing once with high salt buffer (buffer A with 1.05 M NaCl). To isolate protein-bound DNA, filters were incubated for 5 min in heated (65°C) SDS buffer (10 mM Tris pH 7.7, 1 mM EDTA, 1% w/v SDS) followed by centrifugation. The SDS incubation
was repeated once more and the SDS fractions pooled. All fractions (low salt (L), high salt (H),
SDS (S)) were precipitated with 0.7 volumes of isopropanol. After incubation at -20°C, DNA
was pelleted by centrifugation and resuspended in DNA loading buffer (0.005% w/v
bromophenol blue, 5% v/v glycerol) prior to resolution on a 1% w/v agarose gel. Gels were
stained post-electrophoresis with 1 μg ml⁻¹ ethidium bromide.

ATP hydrolysis assay using [γ³²P]-ATP

Topo II-mediated ATP hydrolysis was monitored by thin layer chromatography as previously
described (Kingma et al., 2001) but with the following modifications. Human topo IIα (31 nM)
was incubated in reaction buffer A in the presence of 1.2 μg (6 pmol) pBluescript-KS(+) plasmid
DNA and the indicated drug for 10 min at room temperature prior to initiating the reaction with
the addition of 3 μCi of [γ⁻³²P]-ATP (PerkinElmer; 3000 Ci/mmol). Reactions were incubated at
37°C. At the indicated time intervals, 2 μl aliquots were spotted onto pre-washed (distilled water)
polyethylenimine-impregnated cellulose plates (Sigma-Aldrich) and air-dried. Reaction
products were resolved by developing plates with freshly prepared 400 mM NH₄HCO₃. Plates
were air-dried and exposed to autoradiographic film. Spots corresponding to free phosphate were
excised from the thin layer chromatography plates and quantified using a scintillation counter.
For assays utilizing apyrase, reactions contained 2 milliunits of apyrase in place of topo IIα in
identical buffer conditions, but in the absence of DNA.

Malachite green ATPase assay

To determine the kinetics of topo IIα-mediated ATP hydrolysis, a colorimetric assay utilizing
malachite green and ammonium molybdate was used (Tretter and Berger, 2012). In a 96-well
plate, 50 μl reactions containing topo IIα (8 units) were prepared in reaction buffer A containing varying concentrations of salicylate or novobiocin as well as 2 μg (10.2 pmol) pBluescript-KS(+) DNA. Reactions were initiated by the addition of increasing concentrations of ATP (di-sodium salt, Topogen), covered and incubated at 37°C for 25 min. Immediately following the incubation, 200 μl of malachite green reagent (0.034% w/v malachite green, 3.4% v/v ethanol, 10 mM ammonium molybdate, 1 N HCl) was added and absorbance measured at 650 nm. Reactions examining liberation of phosphate by apyrase were carried out under identical buffer conditions using 2 milliunits of apyrase but in the absence of DNA.

**ATP-independent, pre-strand passage cleavage assay**

Topo IIα or β-mediated cleavage of plasmid DNA in the absence of nucleotide triphosphate and magnesium was carried out as previously described (Bandele and Osheroff, 2009) with the following modifications. Topo IIα or topo IIβ (41.4 nM homodimer) was incubated in a reaction with 200 ng (0.64 pmol) pBR322 with increasing concentrations of salicylate or ICRF-193. The final buffer contained 10 mM Tris pH 7.7, 50 mM NaCl, 50 mM KCl, 0.1 mM EDTA, 5 mM CaCl₂, 0.03 μg μl⁻¹ bovine serum albumin. Reactions were incubated at 37°C for 15 min prior to the addition to of SDS (1% w/v) and continued incubation for an additional 5 min. Reaction were then digested by the addition of 8 μg proteinase K and incubated at 56°C at 30 min. DNA products were resolved on a 1.4% w/v agarose gel containing 0.7 μg μl⁻¹ ethidium bromide.

**Post-strand passage cleavage assay**

Topo IIα-mediated post-strand passage cleavage was measured as described above for pre-strand passage cleavage except that reactions contained 0.5 mM AMPPNP.
ATP-independent DNA religation assay

Topo IIα-mediated religation of plasmid DNA in the absence of a nucleotide triphosphate was carried out as previously described (Osheroff, 1989). Topo IIα (41.4 nM homodimer) was incubated in a reaction with 200 ng (0.64 pmol) pBR322 plasmid DNA in a reaction buffer containing 10 mM Tris pH 7.7, 50 mM NaCl, 50 mM KCl, 0.1 mM EDTA, 5 mM CaCl₂, 0.03 μg μl⁻¹ bovine serum albumin at 37°C for 10 min. Immediately after, samples received either salicylate (to a final concentration of 30 mM) or vehicle, followed by the addition of 2 μl of 100 mM EDTA to all samples. Reactions were then re-initiated by the addition of 2 μl of 100 mM MgCl₂ and transferred immediately to ice. After the indicated time points (15, 30 or 60 sec), reactions were stopped by the addition of SDS (1% w/v) and incubated for an additional 5 min at 37°C. Reactions were then incubated with 8 μg proteinase K at 56°C at 30 min. DNA products were resolved on a 1.4% w/v agarose gel containing 0.7 μg μl⁻¹ ethidium bromide.

kinetoplast DNA (kDNA) assay

The effect of salicylate on topo IIα and topo IIβ catalytic activity was examined as previously described (Bau and Kurz, 2011) with the following modifications. Human topo IIα or topo IIβ (32 nM) were incubated with 200 ng kinetoplast DNA (kDNA, Topogen) in reaction buffer A for 10 min on ice prior the addition of ATP (0.5 mM) and further incubation at 37°C for 8 min. Reactions were stopped by the addition of SDS (1% w/v) and reaction products were resolved on a 1% w/v agarose gel containing 0.7 μg μl⁻¹ ethidium bromide.
Results

Salicylate does not intercalate DNA and does not interfere with topo II-DNA binding

The inhibition of topo II catalytic activity can occur at one of several stages of the catalytic cycle. As a consequence, multiple independent approaches are required to determine the mechanism of drug-mediated inhibition of topo II. Some topo II catalytic inhibitors intercalate DNA, binding either the major or minor groove (Ketron et al., 2012; Larsen et al., 2003); distortion of the DNA backbone can preclude topo II-DNA interaction. The ability of salicylate to intercalate DNA was assessed using a topo I unwinding assay (Pommier et al., 1987). Supercoiled pHOT1 plasmid DNA was relaxed by the addition of topo I. The addition of a known DNA intercalator (mAMSA) (Larsen et al., 2003; Nitiss, 2009b) results in negative supercoiling of the plasmid, which migrates with greater mobility than its relaxed counterpart (Fig. 1A). In contrast, the addition of increasing concentrations of salicylate did not alter the mobility of the relaxed topoisomers, indicating that salicylate does not intercalate DNA (Fig. 1A).

The ability of salicylate to interact with DNA was also assessed using a fluorescence-based ethidium bromide displacement assay (Fortune and Osheroff, 1998). The fluorescence of ethidium bromide is greatly enhanced upon intercalation into DNA, which is quantifiable by fluorescence spectroscopy. As expected, the addition of mAMSA was accompanied by a concomitant decrease in ethidium bromide fluorescence (Fig. 1B), indicative of ethidium bromide displacement. In contrast, the addition of increasing concentrations of salicylate did not
alter the intensity of ethidium bromide fluorescence (Fig. 1C). Together, these findings suggest that salicylate does not inhibit topo IIα catalytic activity by intercalating DNA.

As an alternative approach to examine whether salicylate interferes with the ability of topo II to bind DNA, we utilized EMSAs. Robust topo II-DNA binding was observed when a radiolabeled probe containing a strong topo II binding site was used (Fig. 1D) (Kurz et al., 2000; Peebles et al., 2001). The presence of topo II in this complex was confirmed by both competition assays with unlabeled self and non-specific double-stranded oligonucleotides and by supershift assay using a topo IIα-specific antiserum (Supplemental Fig. 1). Addition of hypericin, a previously established catalytic inhibitor of topo II that blocks DNA binding (Peebles et al., 2001), decreased the topo II-DNA interaction in a dose-dependent manner (Fig. 1D). In contrast, addition of increasing concentrations of salicylate had no effect on either the formation or mobility of the topo II-DNA complex (Fig. 1D), indicating that salicylate does not alter the ability of topo II to bind DNA. To corroborate our findings, we used purified human topo IIα and performed EMSAs using hypericin and salicylate. As demonstrated in Figure 1E, topo II efficiently binds the entire pool of radiolabeled probe; however, this interaction is prevented in the presence of hypericin, but unaffected by the addition of salicylate, confirming our findings using cell extracts (Fig. 1D).

**Topo IIα ATPase activity is decreased in the presence of salicylate**

Many compounds have been reported to inhibit the ATPase activity of topo II (Hu et al., 2006; Qin et al., 2007); some are competitive inhibitors of ATP binding while others indirectly decrease ATPase activity by inhibiting topo II from re-entering the catalytic cycle, thus reducing...
ATP hydrolysis. Using purified human topo IIα, we monitored enzyme-mediated ATP hydrolysis \textit{in vitro} by thin layer chromatography. Novobiocin, a competitive inhibitor of ATP that binds within the ATP-binding pocket, significantly diminished topo IIα-mediated ATP hydrolysis (Fig. 2A). Consistent with previous reports (Chène et al., 2009; Morris et al., 2000), the closed clamp inhibitor ICRF-193 also reduced topo IIα-mediated ATP hydrolysis (Fig. 2A). Addition of salicylate led to a dose-dependent reduction in topo IIα-mediated ATP hydrolysis (Fig. 2B).

To elucidate the nature of salicylate-mediated inhibition of ATPase activity, we quantified the rates of free phosphate generation during topo IIα-mediated ATP hydrolysis. Inhibition by competitive inhibitors of ATP, such as AMPPNP and novobiocin, can be overcome by increases in substrate (ATP) concentration. This is in contrast to non-competitive inhibitors of topo II-mediated ATP hydrolysis, such as sodium orthovanadate and ICRF-193 (Baird et al., 1999; Harkins et al., 1998; Morris et al., 2000). To examine whether salicylate acts in a competitive or non-competitive manner, we utilized the quantitative, colorimetric malachite green assay. In the presence of novobiocin, a competitive inhibitor of ATP-binding, increasing concentrations of ATP reversed inhibition by novobiocin (Fig. 2C). In contrast, even in the presence of increasing ATP concentrations, salicylate-mediated ATPase inhibition was not altered, indicating that salicylate is a non-competitive inhibitor of topo IIα-ATPase activity (Fig. 2D). As salicylate has been reported to inhibit other cellular enzymes (Deng et al., 2001; Yin et al., 1998) and to examine whether salicylate-based inhibition of ATPase activity is more broadly observed, we investigated if salicylate inhibits the ATPase activity of apyrase, an E-type ATPase (Handa and Guidotti, 1996). In contrast to salicylate-mediated inhibition of topo IIα ATPase activity,
Salicylate did not alter apyrase catalytic activity even at high concentrations of drug (Fig. 2E and 2F).

Salicylate does not promote formation of salt-stable topo IIα closed clamps

Bisdioxopiperazines, such as ICRF-193 and ICRF-187, inhibit topo II by trapping the enzyme as a closed clamp on the DNA (Classen et al., 2003; Morris et al., 2000). Similarly, non-hydrolyzable ATP analogs, such as AMPPNP, trap topo II on DNA by binding to the ATP-binding domains to induce N-terminal clamp closure. In both cases, the enzyme is unable to dissociate from DNA after strand passage, preventing it from re-entering the catalytic cycle, and trapping the enzyme in a non-covalent protein clamp on DNA.

Utilizing a filter-binding assay, we measured DNA trapped in topo IIα closed clamps. In this assay, glass-fiber filters that bind protein, but not free DNA, were used. In the absence topo IIα, supercoiled plasmid DNA readily passes through the filter and is collected in the flow-through/low salt wash (Fig. 3; DNA alone, lane L). In the presence of topo IIα and ATP, the plasmid DNA is relaxed but freely passes through the filter and is also collected in the low salt wash. The relaxed DNA is observed as a slower migrating species on the DNA gel (Fig. 3; ATP, lane L). In contrast, in the presence of AMPPNP, topo IIα-mediated DNA relaxation is inhibited, as indicated by the supercoiled DNA observed in lane L of the AMPPNP sample and a portion of the DNA becomes trapped on the filter in a topo IIα closed clamp. This DNA is only liberated following the denaturation of topo II with hot SDS-containing buffer (Fig. 3; AMPPNP, lane S). Although addition of salicylate led to an inhibition of DNA relaxation (Fig. 3; ATP + 10mM sal, lane L as compared to ATP, lane L), consistent with salicylate’s catalytic inhibition of
topo IIα (Bau and Kurz, 2011), salicylate did not stabilize the enzyme in a closed clamp on DNA (Fig. 3; ATP + 10mM sal, lane S).

**Salicylate inhibits the cleavage reaction of topo IIα.**

As our experimental findings indicate that salicylate does not inhibit topo IIα-DNA interaction or trigger the stabilization of a closed clamp on DNA, we hypothesized that salicylate may inhibit topo II at the DNA cleavage step. Previously, we observed that ATP-dependent topo IIα cleavage activity is completely inhibited by 10 mM salicylate. In the presence of ATP, both pre-strand and post-strand passage DNA cleavage are supported, with the reaction heavily favoring post-strand passage DNA cleavage (Burden et al., 2001). In the absence of ATP, topo II binds DNA and establishes a cleavage-religation equilibrium prior to strand passage. Although topo II requires Mg$^{2+}$ for coordination of the cleavage-religation event, in the presence of Ca$^{2+}$ the cleavage-religation equilibrium shifts to favor stabilization of the cleaved complex (Bandele and Osheroff, 2009) allowing for this step to be experimentally isolated. In the presence of Ca$^{2+}$, topo II cleaves but is unable to religate DNA, trapping the enzyme-DNA complex. The removal of topo II by the addition of a denaturant (SDS) to the reaction and digestion of the protein complex, allows for visualization of a linear DNA band, corresponding to the pre-strand passage DNA cleavage event. As ATP is required for strand passage, the absence of ATP in the reaction allows for isolation of the pre-strand passage event. To examine the effects of salicylate on topo IIα pre-strand passage DNA cleavage, we carried out a plasmid DNA cleavage assay in the presence of Ca$^{2+}$ and absence of ATP.

Incubation with increasing concentrations of salicylate led to a dose-dependent loss of cleaved DNA, represented by the loss of the linear DNA band (Fig. 4A and 4B). Furthermore,
we observed that salicylate prevents topo IIα-mediated single-strand DNA cleavage, as shown by the decreased intensity of the slower mobility nicked circular band (Bandele and Osheroff, 2009). ICRF-193, which inhibits enzyme activity post-cleavage and strand passage, had no effect on the formation of cleaved DNA or on levels of nicked circular DNA (Fig. 4A and 4C). Together, these results indicate that salicylate can block the topo II-mediated pre-strand passage DNA cleavage event.

To determine if salicylate has similar effects on post-strand passage DNA cleavage, we repeated the above experiment, but with the inclusion of AMPPNP in the reaction buffer (Robinson and Osheroff, 1991). The addition of this non-hydrolyzable ATP analog permits strand passage to occur. Carrying out the reaction in the presence of Ca\(^{2+}\) thus isolates post-strand passage DNA cleavage. In the presence of AMPPNP and Ca\(^{2+}\), incubation with increasing concentrations of salicylate led to a dose-dependent loss of cleaved DNA, represented by the loss of the linear DNA band (Fig. 4D and 4E). Together, these results indicate that salicylate can block both topo II-mediated pre-strand and post-strand DNA cleavage reactions.

We subsequently examined the effect of salicylate on topo II-mediated DNA religation. Similar to the experiment above, we trapped kinetically competent, topo II-DNA complexes in the presence of Ca\(^{2+}\). After sequestration of the excess Ca\(^{2+}\) ions by the addition of EDTA, re-introduction of Mg\(^{2+}\) allows for religation of the linear band (Osheroff, 1989). To examine whether salicylate impacts the religation of DNA by topo II, we initiated reactions with Mg\(^{2+}\) in the presence or absence of salicylate (Fig. 4F). Our experiments demonstrate that the religation of DNA occurs almost instantaneously and is not impeded in the presence of 30mM salicylate, a concentration that severely impairs DNA cleavage.
Salicylate preferentially inhibits the alpha isoform of human topo II

While most catalytic inhibitors and poisons of human topo II act on both the alpha and beta isoforms, a limited number of compounds have been described that preferentially inhibit one isoform (Auzanneau et al., 2012; Gao et al., 1999; Toyoda et al., 2008). To examine whether salicylate can inhibit the beta isoform of topo II with equal potency, we performed decatenation assays utilizing purified topo IIα and topo IIβ. Strikingly, we found that salicylate preferentially inhibits the activity of the topo IIα isoform, demonstrating complete inhibition of decatenation at 1 mM, whereas no inhibition of the topo IIβ isoform was observed at concentrations up to 10 mM (Fig. 5A). To determine if preferential inhibition was observed when assessing pre-strand passage, we repeated the plasmid cleavage assay in the presence of Ca²⁺ and the absence of ATP with purified human topo IIβ (Fig. 5B and 5C). Similar to the decatenation assay, no inhibition of topo IIβ was observed at concentrations up to 30 mM, although inhibition of topo IIβ was observed at 50 mM salicylate, a suprapharmacologic dose.
Discussion

Compounds that disrupt the catalytic activity of topo II are broadly classed with multiple mechanisms of action (Fig. 6). Topo II poisons (such as doxorubicin and etoposide) stabilize the topo II-DNA cleavable complex leading to an accumulation of DNA double-stranded breaks, thus overwhelming the cell’s capacity for repair. In contrast, catalytic inhibitors inhibit topo II without directly inducing DNA strand breaks. Previously, we identified salicylate as a novel catalytic inhibitor of human topo II (Bau and Kurz, 2011). Importantly, we determined that pretreatment of cells with salicylate attenuates topo II poison-induced DNA double-stranded break formation, thereby decreasing the cytotoxic effects of doxorubicin and etoposide. This effect is specific for topo II and independent of salicylate-mediated inhibition of cyclooxygenases or NF-κB (Bau and Kurz, 2011). Given that salicylate-based therapeutics, including aspirin (acetylsalicylic acid), are widely accessible and widely consumed, we sought to determine the biochemical mechanism underlying the salicylate-mediated inhibition of topo II.

We tested the possibility that salicylate could disrupt the topo II-DNA interaction through distortion of the DNA helical structure (Fig. 1). DNA intercalation is typically observed with compounds such as mAMSA and doxorubicin that contain planar aromatic rings and positive charges, enhancing their interaction with DNA (Armitage, 2005). While salicylate, also known as 2-hydroxybenzoate, contains an aromatic ring, its structure is compact and anionic, thus limiting its potential for direct interaction with DNA. To further corroborate our findings, we
utilized gel shift assays that demonstrated no effect of salicylate on topo II-DNA binding (Fig. 1).

We observed that salicylate decreases topo IIα-mediated ATP hydrolysis (Fig. 2B). This inhibition was non-competitive, as it was not overcome by increasing the concentration of ATP (Fig. 2D); such an effect on topo II has been observed with vanadate and bisdioxopiperazines (Harkins et al., 1998). This is in contrast to competitive inhibitors, such as AMPPNP, salvicine novobioicin (Fig. 2C) and QAP1, where the effectiveness of inhibition is decreased with a corresponding increase in ATP (Chène et al., 2009; Hu et al., 2006). Both competitive and non-competitive inhibitors of topo II ATPase activity can promote the formation of enzyme ‘closed clamps’ (Classen et al., 2003; Harkins et al., 1998; Morris et al., 2000; Vaughn et al., 2005). Compounds that promote topo II-closed clamps permit DNA cleavage, strand passage and religation, but prevent dissociation of topo II from the DNA, thereby trapping the enzyme. However, despite decreasing topo IIα-mediated ATP hydrolysis, the addition of salicylate failed to stabilize topo II in a closed clamp on DNA (Fig. 3), even at high (100 mM) concentrations (unpublished observations). We did, however, observe complete inhibition of topo IIα catalytic activity (Fig. 3; ATP + 10mM sal, lane L), consistent with our earlier work (Bau and Kurz, 2011). Thus, in the case of salicylate and topo II, the apparent non-competitive inhibition of ATPase activity appears to be an indirect effect of inhibition elsewhere in the catalytic cycle.

Cleavage assays performed in the presence of Ca^{2+} and the absence of a nucleotide triphosphate allow for the interrogation of DNA cleavage in the absence of strand passage (Bandele and Osheroff, 2009; Osheroff and Zechiedrich, 1987; Schmidt et al., 2012). Using this
approach, we demonstrate that salicylate inhibits the pre-strand passage DNA cleavage activity of topo IIα. This mechanism of inhibition has previously been observed with two other catalytic inhibitors of topo II: merbarone and staurosporine (Fortune and Osheroff, 1998; Lassota et al., 1996). Although these three agents inhibit the ATP-independent nucleophilic attack of the DNA by the catalytic site tyrosine, inhibition of topo II catalytic activity by salicylate (Fig. 2) and staurosporine (Lassota et al., 1996) is also associated with non-competitive inhibition of topo II ATPase activity. In contrast, merbarone has been reported not to impact topo II ATPase activity (Fortune and Osheroff, 1998), although this is not the case in our hands (Bau & Kurz, unpublished observation). In fact, inhibition of topo II-mediated ATP hydrolysis secondary to inhibition elsewhere in the catalytic cycle has been commonly observed among catalytic inhibitors of topo II (Lassota et al., 1996; Robinson et al., 1993). In addition to inhibition of pre-strand passage DNA cleavage, we also observed salicylate-mediated inhibition of post-strand passage DNA cleavage (Fig. 4D and 4E). Despite the ability of salicylate to impair DNA cleavage both pre- and post-strand passage, the compound does not affect DNA religation, as resealing of the cleaved DNA was almost instantaneous (Fig 4F). Together, these observations indicate that salicylate selectively acts upon DNA cleavage and not at other steps within the enzymatic cycle.

Somewhat surprisingly, we found that salicylate inhibits topo II with at least 10-fold greater selectivity for the alpha isoform (Fig. 5). Previous work has identified topo IIα or topo IIβ specific poisons, with selectivity ranging from 1.5-fold to greater than 10-fold (Bandele and Osheroff, 2007; Gao et al., 1999; Toyoda et al., 2008). Recent work has reported the first isoform selective catalytic inhibitors of topo IIα, polyphenolic ellagitannin derivatives (Auzanneau et al.,
The biochemical basis of isoform selectivity with these compounds and salicylate remains unknown. Human topo IIα and topo IIβ share very similar catalytic activity and are highly conserved, with 78% amino acid identity in the N-terminal three-quarters of the enzyme, but falling to only 34% identity in the C-terminus (Austin and Marsh, 1998). Whether this C-terminal region plays a role in determining the selectivity of salicylate for the alpha isoform may be revealed by further research mapping the region of interaction. Taken together, these studies highlight important findings that may aid in our understanding of the chemical requirements necessary for isoform selective catalytic inhibition of topo II.

There is increasing interest in the identification and development of isoform-selective topo II poisons and inhibitors. This has emerged largely because treatment with topo II poisons is associated with a number of side effects, including cardiomyopathy and secondary malignancies (particularly MLL translocation-driven leukemias) (Azarova et al., 2007; Cowell et al., 2012; Zhang et al., 2012). Recent work has established that poisoning of topo IIβ by these chemotherapeutics drives both cardiotoxicity (Zhang et al., 2012) and development of secondary malignances (Cowell et al., 2012). Interestingly, co-administration with topo II catalytic inhibitors (specifically bisdioxopiperazines) significantly reduces the risk of these side effects in patients (Cvetković and Scott, 2005; Vrooman et al., 2011). However, stabilization of topo II in closed clamps on DNA results in topological blocks that can lead to deleterious cytological defects (chromosomal aberrations, endoreduplication) (Germe and Hyrien, 2005). This shortcoming may be overcome with the development of isoform-specific topo II catalytic inhibitors that do not leave the enzyme trapped on DNA.
Salicylate is a widely used drug that has multiple mechanisms of action. In addition to our work identifying it as a catalytic inhibitor of topo IIα, salicylate is known to inhibit IκB kinase and the heat shock family member glucose-regulated protein 78 (GRP78) (Deng et al., 2001; Yin et al., 1998), while, in contrast, it is an activator of AMP-activated protein kinase (AMPK) (Hawley et al., 2012). Aspirin and related salicylates remain among the most widely used drugs in the world, with an estimated 40,000 metric tons of aspirin consumed annually (Warner and Mitchell, 2002). Although salicylate is not sufficiently potent to be cytotoxic at clinically achievable concentrations, our previous work demonstrated that pretreatment of cells with salicylate attenuates both doxorubicin and etoposide cytotoxicity (Bau and Kurz, 2011). Whether co-administration of salicylate reduces the efficacy of anti-tumor therapy in xenograft model systems remains an ongoing area of investigation. This work may provide justification for discouraging the use of aspirin and related drugs in patients undergoing cancer treatment using topo II poisons.
Authorship Contributions:

Participated in research design: Bau, Kang, Kurz

Conducted experiments: Bau, Kang

Contributed new reagents or analytic tools: Austin

Performed data analysis: Bau, Kurz

Wrote or contributed to writing of the manuscript: Bau, Kurz
References


Footnotes:

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Figure Legends

Fig. 1. Salicylate does not intercalate DNA and does not bind the minor groove of DNA. (A) The topoisomerase I (topo I)-mediated DNA unwinding assay was completed as described in Materials and Methods. pHOT1 plasmid was incubated with topo I prior to the addition of either amsacrine (mAMSA; 10, 50 or 100 μM) or salicylate (1, 3 or 10 mM). Reaction products were resolved on an agarose gel containing 0.2 μg ml⁻¹ chloroquine prior to visualization with ethidium bromide. RLX, relaxed DNA; SC, supercoiled DNA. (B and C) Supercoiled plasmid DNA (5 nM) was incubated in the presence of ethidium bromide (EtBr) and increasing concentrations of mAMSA (B) or salicylate (sal; C). Fluorescence values (excitation 510 nm/emission 595 nm) are plotted as mean fold increase over buffer, with error bars representing the standard deviation from three independent experiments. (D) Topo II-DNA binding is not altered by salicylate but is decreased by a known catalytic inhibitor of topo II-DNA binding, hypericin. MCF-7 nuclear extracts were incubated with a [³²P]-labeled double-stranded oligonucleotide containing a strong topo II binding site (Kurz et al., 2000; Peebles et al., 2001) in the presence of increasing concentrations of hypericin or salicylate. Reaction products were resolved by non-denaturing polyacrylamide gel electrophoresis, dried and exposed to autoradiographic film. (E) Reactions described in (D) were repeated using purified human topo II in the presence of hypericin or salicylate. Binding reactions contained equal moles of binding site and topo II (0.8 fmol) and were incubated at room temperature for 30 min. Reaction products were processed as in (D).
Fig. 2. Salicylate non-competitively inhibits topo IIα ATPase activity. (A) Topo IIα-mediated ATP hydrolysis was monitored by thin layer chromatography using purified human topo IIα in a reaction containing plasmid DNA and [γ\(^{32}\)P]-ATP with 1 mM novobiocin or 0.1 mM ICRF-193. The spots corresponding to free inorganic phosphate were excised and quantified by scintillation counting. (B) Reactions as in (A) were carried out with or without the indicated concentrations of salicylate (sal). (C and D) The detection of free phosphate release during ATP hydrolysis by topo IIα was quantified using malachite green in the presence of either novobiocin (C) or salicylate (D). (E) The detection of free phosphate release by apyrase was quantified using thin layer chromatography as in (A) using 2 milliunits of apyrase. (F) Hydrolysis of free phosphate during ATP hydrolysis by apyrase was examined using malachite green. Reactions were incubated at 25°C for 8 min prior to the addition of the malachite green reagent. Absorbance readings were measured at 650 nm.

Fig. 3. Topo IIα closed clamps are not stabilized by salicylate. To analyze topo IIα closed clamps, filter binding assays were utilized as outlined in the Materials and Methods section. Topo IIα (33 nM) was incubated with 15.5 nM (600 ng) pBluescript DNA in a reaction containing 0.5 mM ATP, 0.5 mM AMPPNP, or 0.5 mM ATP with 10 mM salicylate (sal). Reaction products were incubated on glass fiber filters, centrifuged and washed twice with low-salt buffer (fraction L). Filters were then washed in a high-salt (1.05 M NaCl) buffer (fraction H). Lastly, filters were incubated in a buffer containing 1% SDS (heated to 65°C) for 10 min prior to centrifugation (fraction S). DNA in each fraction was precipitated and recovered DNA products were resolved by agarose gel electrophoresis.
Fig. 4. Salicylate inhibits topo IIα at a step preceding strand passage. (A) Topo IIα-mediated cleavage of pBR322 plasmid was carried out in the absence of ATP and in the presence of Ca²⁺. Plasmid DNA was incubated with topo IIα (10 units) for 15 min at 37°C in the presence of either salicylate (10, 30 or 50 mM) or ICRF-193 (10, 50, 100 μM). Reactions were stopped by the addition SDS prior to digestion with proteinase K. Plasmid DNA was precipitated and resolved on a 1% agarose gel containing 0.7 μg ml⁻¹ ethidium bromide for 15 h at 12V. SC, supercoiled DNA; LN, linear DNA; NC, nicked circular DNA. (B and C) The intensities of the linear bands observed following incubation with salicylate (B) or ICRF-193 (C) were quantified and plotted as percentage of control. The graphs represent the means and standard deviations of three independent experiments. (D and E) The effect of salicylate on topo IIα-mediated post-strand passage DNA cleavage was measured as in (A); however, all reactions included AMPPNP (0.5 mM). Reaction products were processed and visualized as in (A) and the linear (LN) band was quantified and plotted in (E). (F) Topo IIα-mediated religation of pBR322 plasmid was examined in the presence or absence of salicylate. Kinetically-competent topo II-DNA complexes were trapped in the presence of Ca²⁺ in the absence of ATP. After addition of salicylate, reactions were reinitiated with Mg²⁺ and trapped at the indicated time points and examined as in (A). SC, supercoiled DNA; LN, linear DNA; NC, nicked circular DNA.

Fig. 5. Salicylate selectively inhibits the topo IIα isoform. (A) Equimolar concentrations of topo IIα or topo IIβ were incubated with salicylate and 200 ng kDNA for 8 min at 37°C. Reactions were stopped by the addition of SDS and resolved by agarose gel electrophoresis and staining with ethidium bromide. All lanes shown are from the same experiment but are represented with
separating spaces to reflect removal of experimental lanes unrelated to this study. (B) Topo IIβ was incubated in a reaction containing plasmid DNA, 5 mM CaCl₂ and salicylate. Reactions were stopped by the addition of SDS, digested with proteinase K, and resolved overnight on an agarose gel containing 0.7 μg ml⁻¹ ethidium bromide. The DNA products are denoted as follows: NC, nicked circular; LN, linear, SC, supercoiled. The density of the linear bands in (B) were quantified and plotted in (C) as the means and standard deviations from three independent experiments.

Fig. 6. Model for salicylate-mediated inhibition of topo IIα. The catalytic cycle of topo II can be inhibited at any one of several discrete steps. Agents, such as mAMSA, hypericin or aclarubicin, inhibit the binding of topo II to DNA. After topo II binding, cleavage of the double-stranded gate segment of DNA (black) occurs in a Mg²⁺-dependent reaction that can be inhibited by merbarone or salicylate. Trapping of the double-stranded transfer segment of DNA (red) by closure of the N-terminal protein clamp occurs following the binding of two molecules of ATP. Closure of this clamp can be blocked by ATP analogs, such as AMPPNP and novobiocin. The hydrolysis of one ATP molecule triggers the passage of the transfer segment, which is immediately followed by religation of the transient double-stranded break in the gate segment. Chemotherapeutics such as doxorubicin and etoposide poison topo II by preventing gate segment religation. After religation of the gate segment, hydrolysis of the second ATP molecule releases the enzyme from the DNA, allowing it to re-enter the catalytic cycle. Bisdioxopiperazines, such as ICRF-193, prevent the release of topo II from DNA, trapping it as a closed clamp.
Figure 2

A

ATP hydrolyzed (pmol)

Time (mins)

no drug
1 mM novobiocin
0.1 mM ICRF-193

B

ATP hydrolyzed (pmol)

Time (mins)

control
1 mM sal
3 mM sal

C

absorbance

ATP (mM)

control
100 μM novobiocin

D

absorbance

ATP (mM)

control
1 mM sal
3 mM sal

E

ATP hydrolyzed (pmol)

Time (mins)

control
10 mM sal

F

absorbance

ATP (mM)

control
10 mM sal
100 mM sal
Figure 3

The figure shows a gel electrophoresis with lanes labeled L, H, and S for different conditions. The conditions include DNA alone, ATP, AMPPNP, and ATP + 10mM sal.
Figure 6

mAMSA
aclarubicin
hypericin

2 Mg$^{2+}$
salicylate
merbarone

novobiocin
AMP-PNP

2 ATP
doxorubicin
etoposide

ICRF-193

ADP + Pi
Supplemental Data for MOL #88963

Article: Salicylate, a catalytic inhibitor of topoisomerase II, inhibits DNA and is selective for the alpha isoform

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Supplemental Fig. 1. Nuclear extracts were prepared from MCF-7 cells and incubated with a $[\alpha-^{32}\text{P}]$-labeled double-stranded oligonucleotide (~5-10 fmol) containing a strong topo II binding site (Kurz et al., 2000; Peebles et al., 2001). *Left panel:* To demonstrate sequence-specificity, binding reactions were carried out in the presence of increasing molar excesses of unlabeled binding site (self) or an unrelated unlabeled double-stranded oligonucleotide (non-specific). *Right panel:* The presence of topo II$\alpha$ in the observed binding complex was confirmed by supershift assay with the addition of either a topo II$\alpha$-specific antiserum (topo II ab) or a pre-immune control antiserum (non-spec ab). The two supershift panels are from the same experiment, but are represented with a space between them to reflect the removal of experimental lanes unrelated to this study. For all experiments, reaction products were resolved by non-denaturing polyacrylamide gel electrophoresis, dried and exposed to autoradiographic film.