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Characterization of Salicylates as Novel Catalytic Inhibitors of Human DNA Topoisomerase II Alpha

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Characterization of Salicylates as Novel Catalytic Inhibitors of Human DNA Topoisomerase II Alpha

by

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Abstract

Topoisomerase II (topo II) is a ubiquitous enzyme required for the maintenance of DNA topology. Due to its essential nature, many pharmaceuticals have been found to or developed to target the enzyme for both laboratory and clinical uses. Topo II poisons, such as doxorubicin and etoposide, are commonly used in cancer chemotherapy regimens and are cytotoxic due to their ability to covalently trap topo II on cleaved DNA, leading to the accumulation of DNA double-stranded breaks. In contrast, compounds that inhibit topo II catalytic activity without generating DNA double-stranded breaks are classified as catalytic inhibitors and impair enzyme function at a number of stages in the catalytic cycle, including DNA binding, DNA cleavage, ATP hydrolysis, and enzyme dissociation. Some catalytic inhibitors have been found to counter the DNA damaging effects of topo II poisons.

This thesis describes work identifying salicylate as a novel catalytic inhibitor of topo II. It demonstrates that pretreatment of cells with salicylate, prior to treatment with topo II poisons, attenuates poison-induced DNA damage signaling mediated by the ATM protein kinase. This is associated with a concomitant loss of poison-induced DNA double-stranded breaks and consequently decreased cytotoxicity following treatment with topo II poisons. This work also demonstrates that salicylate does not block topo II-DNA binding, or trap the enzyme as a closed clamp on DNA. Rather, salicylate blocks DNA cleavage and enzyme-mediated ATP hydrolysis is impaired in a non-competitive manner. Furthermore, salicylate-mediated inhibition of topo II catalytic activity is selective for the alpha isoform of topo II.

In investigating the structural determinants modulating potency of topo II inhibition, many compounds with structural similarities to salicylate, including the salicylate-based pharmaceuticals sulfasalazine and diflunisal, were found to also act as catalytic inhibitors of topo II. It was determined that potency is determined by modifications at the 2'-position that are electronegative and capable of donating a hydrogen bond, but that these effects are further influenced by substitutions at the 5'-position. Considered together, the data presented in this thesis establish a new role for a long-established drug.

Preface

Contributions to thesis work:

Jason Bau performed, analyzed and interpreted the majority of the experiments with the support of his supervisor, **Dr. Ebba Kurz**. **Ms. Zhili Kang** performed the experiment examining the selective effects of salicylate on topo II alpha and beta. **Dr. Caroline Austin** (Newcastle University, United Kingdom) provided purified topo II beta required for completion of the experiments.

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Dedication

To my family, my parents, Andrew and Renata, and my brother, Jeremy.
Thank you for your unconditional love and support.

To my beautiful partner in life, Ting.
I love you more than spaghetti loves spaghetti sauce
and I dedicate this work to you.

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List of Symbols, Abbreviations and Nomenclature

Symbol	Definition
Units of Measure	
%	percent
°C	degrees Celsius
g	gram
mg	milligram
µg	microgram
x g	gravitational acceleration (1 g = 9.81 m/s^2)
hr	hour
kDa	kilodalton
M	molar (moles per litre)
min	minute
mL	millilitre
µL	microlitre
mM	millimolar
µM	micromolar
µm	micrometre
U	enzyme unit
V	volt
v/v	mL solute per 100 mL solution
w/v	g solute per 100 mL solution
w/w	g solute per 100 g solution
Materials & Methods	
5-IDNR	5-iminodaunorubicin
ADP	adenosine diphosphate
AMPPNP	adenosine 5'-(β,γ-imido)triphosphate
APS	ammonium persulfate
ASA	acetylsalicylic acid
Ca^{2+}	calcium
CAT	catenated kDNA
DCT	decatenated kDNA
DEM	diethyl maleate
DIF	diflunisal
DMEM	Dulbecco's modified Eagle's media
DMSO	dimethyl sulfoxide
DOX	doxorubicin
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
FBS	fetal bovine serum
ICE	*in vivo* complex of enzyme
IR	ionizing radiation
K2	topoisomerase IIα-specific rabbit polyclonal antiserum

kDNA	kinetoplast DNA
MCF-7	Michigan Cancer Foundation human breast cancer cell line
Mg^{2+}	magnesium
MTX	mitoxantrone
NAC	N-acetyl cysteine
NC	nicked circular plasmid DNA
NET-N	NaCl\EDTA\Tris\NP-40 cell lysis buffer
NP-40	nonyl phenoxylpolyethoxylethanol
PBS	phosphate-buffered saline
PMSF	phenylmethanesulfonylfluoride
RLX	relaxed plasmid DNA
SC	supercoiled plasmid DNA
SDS	sodium dodecyl sulfate
SSZ	sulfasalazine
PAGE	polyacrylamide gel electrophoresis
TBS-T	Tris-buffered saline with Tween-20
TEMED	tetramethylethylenediamine
TLC	thin layer chromatography
TPT	topotecan
Tris	tris(hydroxymethyl)aminomethane
VP-16	etoposide

Proteins & Nucleic Acids

53BP1	p53 binding protein 1
APC	anaphase promoting complex
ATM	ataxia telangiectasia mutated
ATP	adenosine triphosphate
ATR	ATM and rad3-related
BSA	bovine serum albumin
Chk2	checkpoint kinase 2
COX-2	cyclooxygenase-2
DNA	deoxyribonucleic acid
DNA-PKcs	DNA-dependent protein kinase, catalytic subunit
dsDNA	double-stranded DNA
HDAC	histone deacetylase
IκBα	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
IKK	inhibitor of kappa B (IκB) kinase
mRNA	messenger RNA
MDC1	mediator of the DNA damage checkpoint 1
MLL	myeloid/lymphoid or mixed-lineage, leukemia
MRN	meiotic recombination 11-Rad50-Nijmegen breakage syndrome 1 complex
Nbs1	Nijmegen breakage syndrome 1
NFκB	nuclear factor kappa B
p	phosphate
pX	phosphorylation at site X (i.e. pSer15)

p53	p53 tumor suppressor
PARP	poly (ADP-ribose) polymerase
PIKKs	phosphoinositide 3-kinase-like kinases
Plk1	polo-like kinase 1
RNA	ribonucleic acid
Ser	serine
ssDNA	single-stranded DNA
TDP2	tyrosyl-DNA phosphodiesterase 2
Thr	threonine
TNFα	tumor necrosis factor alpha
topo I	topoisomerase I
topo II	topoisomerase II
topo IIα	topoisomerase IIα
topo IIβ	topoisomerase IIβ
γ-H2AX	gamma-histone H2AX

General

3’-OH	3'-hydroxyl group
5’-OH	5'-hydroxyl group
C-terminal	carboxy-terminal
DHBA	dihydroxybenzoate
DSB	double-stranded break
G-segment	gate-segment
GHKL	DNA gyrase, Hsp90, histidine-like kinase, DNA mismatch MutL
HBA	hydroxybenzoate
HR	homologous recombination
ICRF-193	4-[2-(3,5-dioxo-1-piperazinyl)-1-methylpropyl]piperazine-2,6-dione
N-terminal	amino-terminal
NHEJ	non-homologous end-joining
NLS	nuclear localization signal
NSAID	non-steroidal anti-inflammatory drug
SUMO	small ubiquitin-related proteins
T-segment	transit-segment
THBA	trihydroxybenzoate
Toprim	topoisomerase-primase

Standing on our microscopic fragment of a grain of sand, we attempt to discover the nature and purpose of the universe which surrounds our home in space and time.

- James Jeans

Chapter One: Introduction

1.1 DNA topology and supercoiling

A key tenet of biology is that organisms must accurately replicate and evenly pass their genetic material from one generation to the next (Meselson and Stahl, 1958; Watson and Crick, 1953). This genetic information must be faithfully packaged, which occurs through highly coordinated and structured processes. These processes require the function of a multitude of enzymes, each with specialized roles.

The packaging of DNA is itself a complex and intricate task. A normal human cell contains roughly 3.2×10^9 base pairs of DNA, enough to stretch almost two metres if placed end-to-end. In contrast, the typical nucleus of a human cell has a diameter of approximately 6 μm. Thus, a cell must have the capability not only to compact its genetic material by many orders of magnitude, but also to relax the compacted DNA to allow for replication and gene expression.

DNA structural organization is hierarchical (Gilbert and Allan, 2013; Koster et al., 2010; Schoeffler and Berger, 2008). Primary structure refers to a linear fragment of nucleotides, while secondary structure describes interactions between strands of DNA (the base pairing found between DNA strands). Tertiary structure describes double-stranded DNA winding upon itself to form the DNA helix, and sections of the helix coiling to form a DNA supercoil. This compaction of long strands of DNA into supercoils describes a further level of genetic packaging. Furthermore, tertiary DNA structure can also be used to describe the intertwining of two separate DNA helices (for example, immediately after

DNA synthesis), forming what are termed DNA catenanes (Schoeffler and Berger, 2008; Witz and Stasiak, 2010).

The process of supercoiling generates two forms of coils: plectonemes or toroids (Fig. 1.1). Toroids are single DNA loops that cross at one point and may be spaced out along a section of DNA. Plectonemes involve the continual twisting of a toroid, resulting in the formation of multiple crossovers. Either structure can be positively or negatively wound. Positive supercoils represent an overwound DNA state and generally are formed ahead of the unwinding helix during DNA replication and gene expression (Gilbert and Allan, 2013; Schoeffler and Berger, 2008).

The DNA superstructures that occur behind the replication fork differ from those formed near the transcriptional machinery. During transcription, negative supercoils are formed trailing the transcription bubble as the result of an underwound DNA state, while DNA replication leads to the generation of new daughter strands of DNA (Gilbert and Allan, 2013; Nitiss, 2009a; Schoeffler and Berger, 2008). Due to the proximity of these daughter strands, as well as the twisting motion of the parental DNA helix, these strands can intertwine each other, forming DNA precatenanes. While DNA supercoils can form topological blocks to replication and transcription, catenanes must be resolved prior to mitosis to allow successful separation of sister chromatids into the daughter cells. Thus, while supercoiling allows for ultra-compaction of the genetic material into the confines of the nucleus, unwinding of the DNA helix is necessary for cells to transmit and express the message stored within the genetic code. However, this process is not without consequence, as unwinding of the DNA helix leads to changes in the topological landscape of DNA (Roca, 2009).

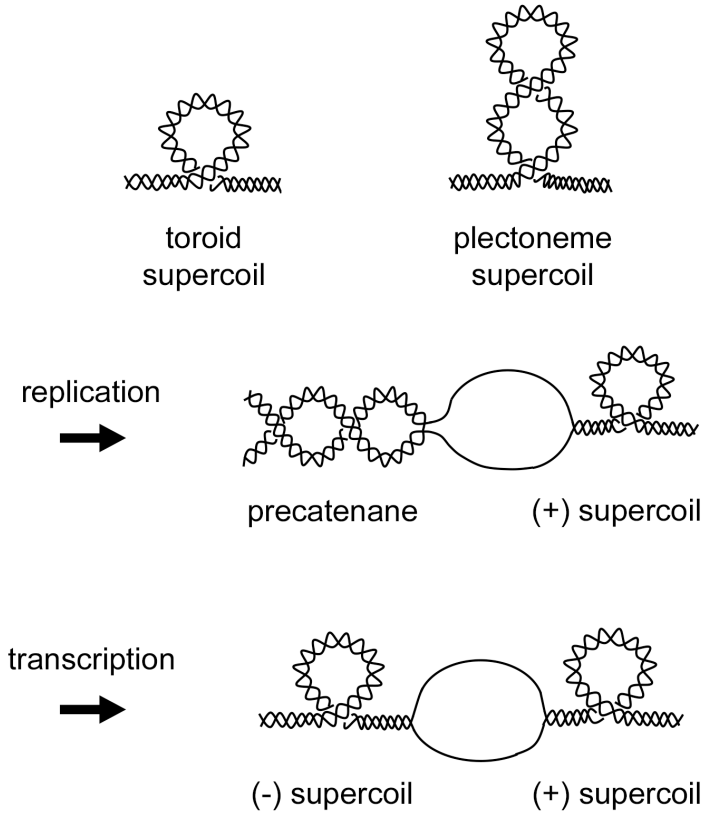

Figure 1.1. DNA supercoiling occurs as a consequence of replication and transcription and takes on multiple forms.

As DNA is unwound and the complementary strands are separated, the tension that is accumulated in the helix emerges as supercoils. Supercoils can exist as either toroids or plectonemes and conversion between these two structures can occur. DNA replication results in the formation of positive supercoils ahead of the replication fork while DNA catenanes form due to the intertwining of the newly synthesized DNA strands behind the replication fork. Transcription also results in positive supercoils ahead of the transcription bubble, with negative supercoils trailing the transcriptional machinery.

1.2 Topoisomerases and their role in DNA packaging

To relieve the topological stresses imparted by these DNA structures, all cells express a family of enzymes with very specialized functions: the topoisomerases (Vos et al., 2011; Wang, 2002). In the broadest sense, the role of topoisomerases is to induce transient breaks in the DNA, thus allowing for relaxation of DNA supercoils and removal of catenanes (Nitiss, 2009a; Schoeffler and Berger, 2008; Wang, 1996). There are several classes of topoisomerases. At the highest level, topoisomerases can be separated into two classes: type I and type II; however, each class is further differentiated based on a number of characteristics (Table 1.1). These characteristics include energy and cofactor requirements, substrate preferences (i.e., recognition of positive or negative supercoils), the nature of the DNA-end modification and differences in unlinking mechanism (Champoux, 2001; Vos et al., 2011). Regardless, all organisms, whether prokaryotic or eukaryotic, express at least one type I and one type II topoisomerase (Forterre and Gadelle, 2009; Gadelle et al., 2003). Certain viruses also appear to encode their own topoisomerases (either type I or type II) and several of these isoforms have been described to have unique properties such as sequence specificity (Minkah et al., 2007; Shuman, 1991), and enhanced cleavage activity (Fortune et al., 2001).

Type I topoisomerases generate single-stranded DNA breaks; these breaks allow the enzyme to relax DNA by one of two mechanisms (Brown and Cozzarelli, 1981; Champoux, 2001; Chen et al., 2013; Pommier et al., 2010; Schoeffler and Berger, 2008). Type IA topoisomerases pass the unbroken DNA strand through the opening in the other strand, prior to resealing of the nick. The type IA topoisomerases are typically found in prokaryotes and require cation co-factors. Type IB topoisomerases also

Table 1.1. Classification of major topoisomerase classes

Type[1]	Subclass	Examples	DNA end linkage	Supercoil affinity	Co-factors
Type I	Type IA	Bacterial & archaeal topo I and III; eukaryotic topo III	5'-phospho-tyrosyl linkage	Negative	Mg^{2+}, Zn^{2+}; Reverse gyrase requires ATP
	Type IB	Eukaryotic topo I; poxvirus topo I	3'-phospho-tyrosyl linkage	Positive & Negative	None
	Type IC	*Methanopyrus kandleri* topo V	3'- phospho-tyrosyl linkage	Positive & Negative	None
Type II	Type IIA	Eukaryotic, viral, bacteriophage topo II; bacterial, archaeal, chloroplast DNA gyrase; bacterial topo IV	5'- phospho-tyrosyl linkage	Positive & Negative	Mg^{2+}, ATP
	Type IIB	Archaeal, plant & algal topo VI	5'-phospho-tyrosyl linkage	Positive & Negative	Mg^{2+}, ATP

[1] The information in this table was gathered from the following references: Schoeffler and Berger, 2008; Nitiss, 2009; Vos et al., 2011

induce single-stranded DNA breaks and, through a controlled rotation or 'swivel' mechanism, rotate the nicked DNA strand around the unbroken strand. The covalent nick generated by bacterial and archeal topoisomerase I (topo I) and topoisomerase III (topo III), as well as eukaryotic topo III is linked to DNA through a 5'-phosphotyrosyl linkage, whereas eukaryotic topo I (type IB) generate phosphotyrosyl linkages with the 3'-end of the cleaved DNA. The type IB class, which includes human topo I, does not require energy or ion co-factors. Importantly, mammalian topo I and topo III have characterized roles in DNA replication and transcription, including removing topological blocks as well as resolving Holliday junctions that arise during recombination (Plank et al., 2006; Wu and Hickson, 2003). Type IB topoisomerases are also the cellular target of a number of widely used anti-cancer chemotherapeutics (Pommier, 2006, 2009). These agents block the enzyme after DNA scission, leading to the accumulation of single-stranded DNA breaks in cells.

Type II topoisomerases relax DNA superstructures through a mechanism distinct from their type I counterparts (Nitiss, 2009a; Schoeffler and Berger, 2008); these enzymes induce transient double-stranded DNA breaks. While remaining covalently bound to the cleaved DNA, the enzyme passes a second double-stranded DNA molecule through the break. Immediately following strand passage, the broken ends are resealed prior to enzyme dissociation from DNA. Unlike the biochemical diversity seen with the type I topoisomerases, both type IIA and type IIB enzymes create covalent 5'-phosphotyrosyl linkages with DNA. Both subtypes require ATP as an energy cofactor, as well as the presence of the divalent cation (Mg^{2+}), and each can catalyze the unwinding of both positive and negative supercoils (Vos et al., 2011). Despite these conserved features, the type IIA and type IIB topoisomerases are genetically distinct, leading to a modest but

important difference in the strand passage mechanism between these two classes (Gadelle et al., 2003).

Type IIA enzymes utilize a two-gate mechanism, trapping DNA in the N-terminal gate, cleaving DNA with the catalytic core, and releasing the passed strand through the C-terminal gate. This mechanism is characteristically seen in type IIA homologs found in yeast (Top2), bacteria (DNA gyrase) and mammalian cells (topoisomerase II (topo II)). In contrast, type IIB topoisomerases employ a one-gate mechanism. While the N-terminal gate is used to trap the double-stranded DNA molecule, the catalytic core that cleaves DNA doubles as the C-terminal gate. Thus, unlike its type IIA counterpart, the type IIB enzymes do not trap the DNA molecule following strand passage. The type IIB topoisomerases represent an evolutionarily distinct class with members identified in plants, archaea and algae; thus far, the only known member of this class is topoisomerase VI (Corbett and Berger, 2003; Gadelle et al., 2003).

Diversity exists amongst type IIA topoisomerase isoforms (Forterre and Gadelle, 2009; Gadelle et al., 2003). While lower eukaryotes, such as yeast (*S. cerevisiae*, *S. pombe*) and flies (*D. melanogaster*), express one type IIA topoisomerase, higher eukaryotes express two isoforms, denoted α and β. In humans, these are encoded by *TOP2A* and *TOP2B*, respectively. In humans, the two isoforms share approximately 70% sequence similarity (Austin and Marsh, 1998); however, much of this occurs within the N-terminal three-quarters of the enzyme (78% identity) with the majority of the divergence occurring in the C-terminal region (34% identity). This region has been proposed to be important for dictating affinity of the enzyme for DNA (Gilroy and Austin, 2011), as well as accounting for the observed preference for positive supercoils by the topo IIα isoform (McClendon et al., 2005, 2008). Moreover, the C-terminal region of topo IIα has been demonstrated to

play an important role in facilitating protein-protein interactions (for example with PCNA) and serves as a site for numerous post-translational modifications (Niimi et al., 2001). The isoforms differ modestly in molecular mass, with the topo IIβ isoform (180 kDa) slightly larger than the topo IIα isoform (170 kDa). While the two isoforms have similar catalytic activities, each plays distinct roles in the modulation of DNA topology, with the topo IIα isoform important for cell division and the topo IIβ isoform important for gene regulation in both proliferating and post-mitotic cells (Champoux, 2001; Nitiss, 2009a).

1.3 Catalytic mechanism of topoisomerase II

Type IIA topoisomerases induce transient DNA double-stranded breaks in order to relax tertiary DNA superstructures. This induction of DNA breaks is exploited for anti-microbial and anti-cancer chemotherapy (Nitiss, 2009b; Pommier, 2013; Pommier et al., 2010). Specifically, targeting of the type IIA topoisomerases (bacterial DNA gyrase, bacterial topo IV, and human topo II) has been and remains a clinically effective approach to treatment (Pommier et al., 2010). Understanding the catalytic cycle of topo II is critical for understanding the mechanisms of action of these agents. While the catalytic mechanism across type IIA topoisomerases is similar, the remainder of this thesis focuses on the human topo II isoforms.

The initiating step of the topo II catalytic cycle is enzyme-mediated recognition of a supercoiled DNA substrate (Figure 1.2). While topo II recognizes DNA independent of its sequence, sites with strong topo II binding affinity and preferred cleavage

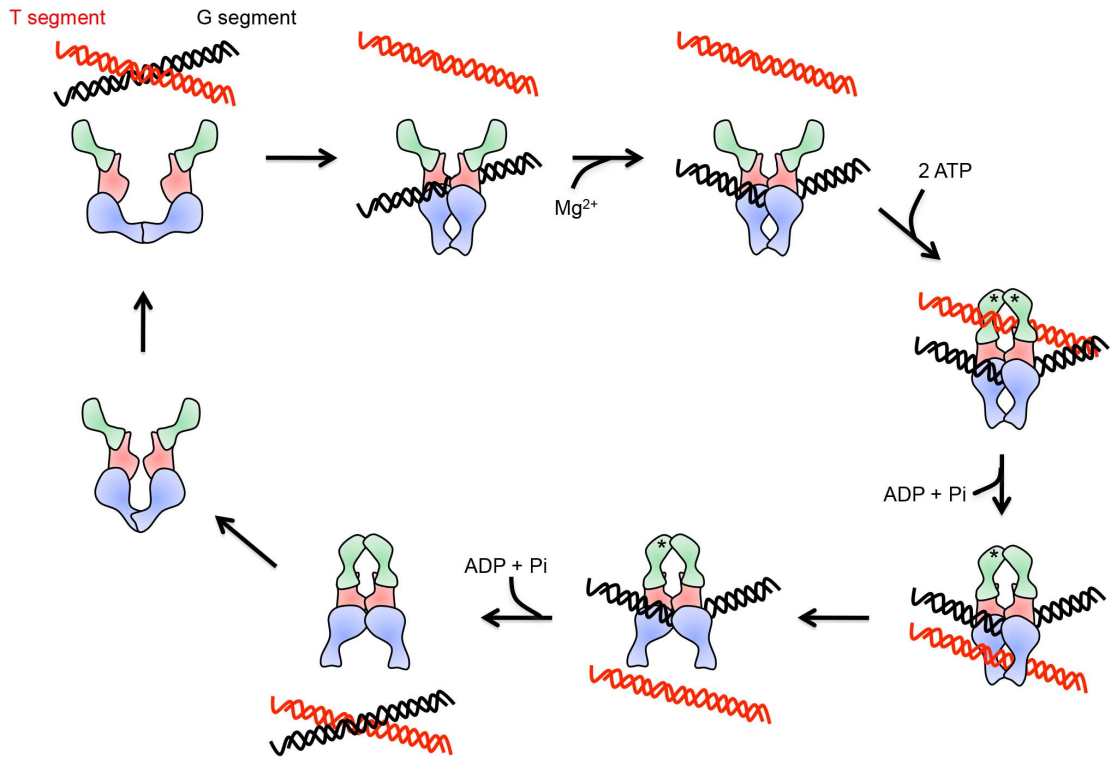

Figure 1.2. The catalytic cycle of topoisomerase II.

Topo II unwinds supercoiled DNA or separates intertwined DNA molecules by first binding to the gate (G)-segment (black double-stranded DNA molecule) within the catalytic core (red) and inducing a DNA double-stranded break. This process requires two Mg^{2+} ions to coordinate the cleavage reaction. The transit (T)-segment (red double-stranded DNA molecule) is then trapped in the N-terminal protein clamp (green), which contains the ATPase domain. The hydrolysis of one ATP molecule (*) results in the passage of the T-segment through the newly formed DNA break in the G-segment and into the C-terminal clamp (blue). The G-segment is resealed and hydrolysis of the second ATP molecule triggers dissociation of the enzyme from the DNA. The enzyme is then free to relax other DNA supercoils or catenanes (adapted from Bau et al., 2014).

have been identified (Capranico and Binaschi, 1998; Mueller-Planitz and Herschlag, 2007; Thomsen et al., 1990). It has been previously speculated that the chemical reactivity between the enzyme and DNA (i.e., that which is more energetically favorable) helps dictate the site for gate (G)-segment cleavage (Bromberg et al., 2002; Mueller-Planitz and Herschlag, 2007). More recently, it has been proposed that topo II recognizes DNA through the higher order structures of DNA, which then determine whether topo II relaxes supercoiled DNA or decatenates intertwined DNA structures (Baxter et al., 2011; Witz et al., 2011). This property of topo II is extremely important, as it has been proposed that topo II preferentially decatenates intertwined DNA over relaxing supercoiled structures. Indeed, this appears to occur with both prokaryotic and eukaryotic type IIA topoisomerases, highlighting the importance of the physical structure of DNA in determining the site of topoisomerase activity (Baxter et al., 2011; Martínez-Robles et al., 2009).

Intersecting double-stranded DNA molecules (whether catenated or supercoiled) are denoted as either the G-segment, or transit (T-) segment. The G-segment is the segment that is cleaved by topo II during the catalytic cycle, thus providing the 'gate' through which the T-segment can pass (Roca and Wang, 1994; Roca et al., 1996). Studies have demonstrated that the enzyme affinity for the G-segment is greater than that of the T-segment (Liu and Wang, 1978; Zechiedrich and Osheroff, 1990). Binding of the G-segment occurs in the breakage-reunion domain of topo II. Once bound, the enzyme bends DNA while cleaving the strand in an Mg^{2+}-dependent reaction (although other divalent metal ions can substitute for Mg^{2+}), which occurs within the topoisomerase-primase (TOPRIM domain of the enzyme (Figure 1.3) (Aravind et al., 1998; Lee et al., 2013, 2012b).

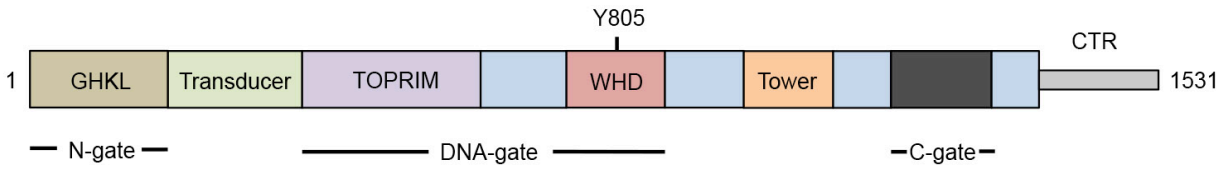

Figure 1.3. The domain map of topoisomerase II alpha

Human topoisomerase II alpha is 1531 amino acids in length and consists of multiple domains required for enzymatic activity. The N-terminal DNA clamp (N-gate) contains the ATPase domain (GHKL; taupe) of topo II. This is adjacent to the Transducer domain (green), which is necessary for relaying the status of the clamp to the DNA-gate. The DNA-gate contains several critical domains, including the TOPRIM domain (purple), necessary for binding of the divalent metal ion, and the winged-helix domain (WHD; red), containing the catalytic tyrosine residue (Y805). The Tower domain (orange) is believed to aid in DNA binding while supporting the cleavage mechanism of the enzyme. Lastly, once the T-segment of DNA has been passed through the DNA-gate, it is trapped within the C-terminal DNA clamp (C-gate; black). The extreme C-terminus of the enzyme (CTR, grey) contains other important sites such as the nuclear localization and nuclear export sequences and is the site of many post-translational modifications.

This cleavage reaction is the hallmark of enzyme function (Liu et al., 1983; Tse et al., 1980). Each topo II protomer contains a catalytic tyrosine residue (Tyr805 and Tyr821 in human topo IIα and topo IIβ, respectively) that mounts a nucleophilic attack of the phosphodiester DNA backbone, thus forming a covalent phosphotyrosine bond with the 5'-end of the DNA (Figure 1.4). Specifically, the free electron pairs of the hydroxyl group of tyrosine attack the electro-positive phosphate atom in the DNA helix (Deweese and Osheroff, 2009; Schmidt et al., 2010). The coordinating Mg^{2+} ions stabilize the protein-DNA transition state in conjunction with neighbouring amino acid residues. While one metal ion directly coordinates the nucleophilic attack by the catalytic tyrosine residue, the second metal ion stabilizes the DNA helix within the catalytic core (Deweese et al., 2008; Schmidt et al., 2010). Additionally, the energy required for cleavage, and later religation, is stored within the newly formed phosphotyrosyl bonds. Thus, the cleavage/religation reaction itself does not require the presence of an energy cofactor (ATP), but rather this cofactor is required for strand passage and enzyme dissociation.

Cleavage of the G-segment by the topo II homodimer results in four base-pair 5'-overhangs and cleavage of the G-segment can occur in the absence of the T-segment (Liu et al., 1983; Schoeffler and Berger, 2008). Additionally, this cleavage event is not unidirectional, but exists in equilibrium between DNA cleavage and religation. As this equilibrium occurs prior to passage of the T-segment, the cleavage event is referred to as pre-strand passage DNA cleavage.

Following cleavage of the G-segment, the enzyme traps the T-segment within the N-terminal protein clamp (Dong and Berger, 2007). The N-terminal clamp has several significant domains that are required for full activity of topo II. Within the N-terminal clamp lies the conserved GHKL ATPase-domain, a conserved ATPase domain in the

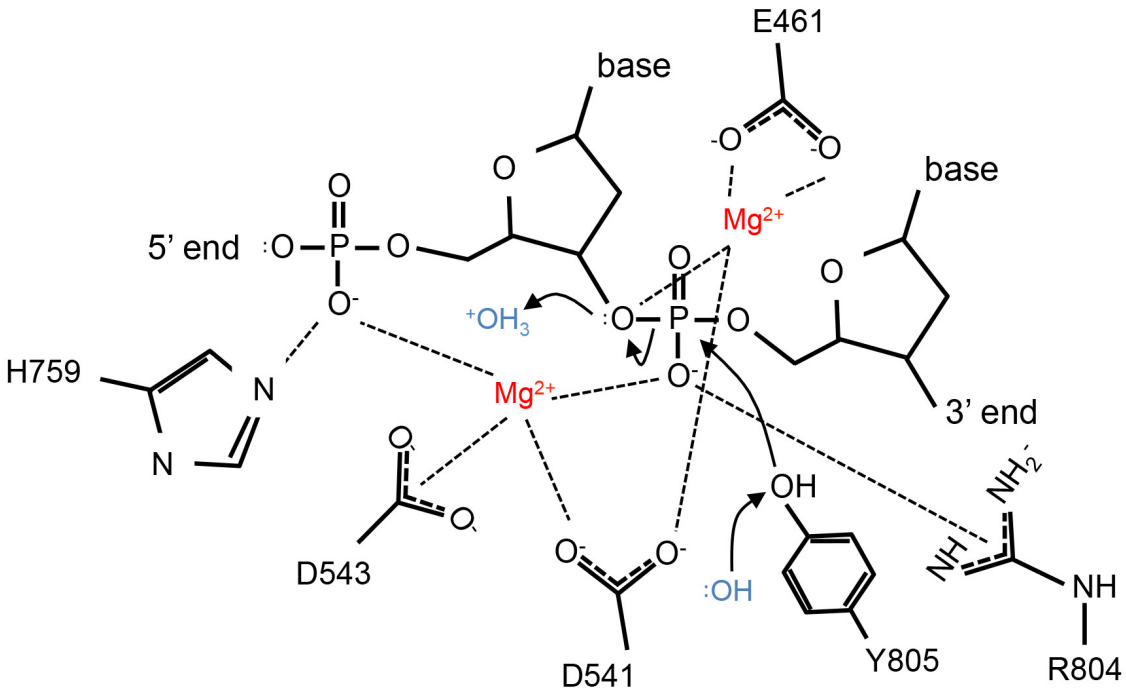

Figure 1.4. Two metal mechanism of topoisomerase II DNA cleavage

The catalytic tyrosine residue (Y805 in topo IIα) initiates a nucleophilic attack of a phosphate group in the DNA backbone. This attack results in the formation of a covalent bond between the tyrosine residue and the 5'-end of the DNA, leaving a free 3'-OH group on the adjacent DNA base. Topo II utilizes two divalent metal ions (Mg^{2+}), which stabilize the cleavage transition state with a number of neighbouring amino acids through non-covalent bonding interactions (topo IIα: E461, D541, D543, H759, R804). Figure adapted from Deweese and Osheroff, 2010.

superfamily of DNA gyrase, the chaperone Hsp90, CheA-type histidine kinases, and the DNA mismatch repair enzyme MutL. As the name suggests, the ATPase domain is responsible for topo II-mediated ATP hydrolysis. Each protomer of topo II contains a single binding site for ATP and binding of two ATP molecules to the homodimer triggers a significant molecular transition – dimerization of the N-terminal clamp. Full activity of the topo II ATPase domain is dependent on binding both G and T-segments of DNA (Corbett et al., 1992; Lee et al., 2012b; Mueller-Planitz and Herschlag, 2006).

The binding of ATP, dimerization of the N-terminal clamp, and passage of the T-segment are interconnected. Specifically, the γ-phosphate of ATP forms a non-covalent interaction with a three amino acid loop (the QTK (glutamine-threonine-lysine) loop) resulting in dimerization of the N-terminal clamp (Bendsen et al., 2009; Bjergbaek et al., 2000). The N-terminus of the enzyme also contains the transducer domain of topo II, which is most accurately described as the inward facing 'walls' of the protein clamp. After ATP binding, this domain (and the space in which the T-segment resides) begins to narrow. Initially, dimerization traps the T-segment of DNA; however, the inner space of the clamp becomes physically constrained by the presence of the DNA molecule. Simultaneously, the G-segment separates to allow for the traverse of the T-segment through the newly formed gate and into the awaiting C-terminal clamp. While this strand passage can occur in the absence of ATP hydrolysis, the rate of passage is promoted by hydrolysis of one of the bound ATP molecules (Lindsley and Wang, 1993). Thus, the transducer domain has a critical role in linking the ATPase domain with the catalytic core to facilitate strand passage (Oestergaard et al., 2004). Highlighting its importance, mutations in this domain that disrupt communication between ATP-binding and DNA cleavage show significant impairment of catalytic activity (Bendsen et al., 2009). At this point, the G-segment

remains cleaved; however, the T-segment is now trapped in the C-terminal clamp. While the cleavage event is not dissimilar from that seen prior to strand passage, a new equilibrium is reached, resulting in what is termed the post-strand passage cleavage event (Robinson and Osheroff, 1991).

Once the T-segment has traversed the double-stranded break induced in the G-segment, the broken segment must be religated prior to enzyme dissociation. Energy for the religation of the G-segment is conserved within the phosphotyrosyl bond. Early studies demonstrated that the religation of the broken DNA occurs sequentially; in other words, one strand is ligated prior to the next (Osheroff and Zechiedrich, 1987; Zechiedrich et al., 1989). The religation occurs in reverse order of the cleavage reaction and still requires the divalent metal ions used in the forward reaction.

Following religation, the enzyme remains associated with DNA, although it no longer remains covalently linked. However, the T-segment remains trapped in the C-terminal clamp and hydrolysis of the remaining ATP molecule triggers opening of this clamp (Osheroff, 1986; Roca and Wang, 1994). At this point, whether simultaneously or sequentially, the resealed G-segment dissociates from the enzyme. The final product is intact G- and T-segments of DNA that have been interchanged within the spatial confines of the nucleus. After dissociation, topo II can re-enter the catalytic cycle.

1.4 Biological roles of the mammalian topoisomerase IIα and topoisomerase IIβ

The biological roles of topo IIα and topo IIβ differ within the cell (Table 1.2). While the role of topo IIβ is less well characterized than topo IIα, studies have identified

Table 1.2. Comparison of human topoisomerase IIα and topoisomerase IIβ

	Topo IIα	Topo IIβ
Molecular mass[1]	170 kDa	180 kDa
Chromosome location	17q21-22	3p24
Catalytic residue	Y805	Y821
Tissue distribution	Proliferating cells	All cells
Expression during cell cycle	Increase from G_1 through S; peak at G_2/M	Does not change through cell cycle
Necessary at cellular level	Yes	No
Essential for viability	Yes, failure at 4-8 cell stage	Cell replication normal; null animals die at birth
Supercoil preference	Positive > negative (10-fold)	No preference
Function	Chromatid separation, DNA relaxation during replication, relaxation of centromeres and telomeres[2]	Regulated transcription, neural development[3]

[1] The information presented in this table was gathered from the following references: Schoeffler and Berger, 2008; Nitiss, 2009; Vos et al., 2011
[2] Germe et al., 2009; Ye et al., 2010
[3] Ju et al., 2006; Rampakakis et al., 2009

critical roles for topo IIβ in regulated transcription and neural development as it is expressed in both proliferating and post-mitotic cells (Ju et al., 2006; Yang et al., 2000). The function of the topo IIα isoform is associated with actively replicating cells, and is essential for the processes of DNA replication and mitosis. An in-depth discussion of the roles of each isoform follows below.

1.4.1 Topoisomerase IIβ: Roles for development and gene transcription

Unlike its topo IIα counterpart, the expression of topo IIβ is independent of cell cycle phase and the enzyme does not associate with DNA during mitosis. While earlier studies examined the cellular distribution of topo IIβ, finding it to be localized to the nucleolus and near heterochromatic regions (Christensen et al., 2002; Zini et al., 1994), recent work has identified a distinct fraction of the enzyme localized within heterochromatin (Cowell et al., 2011). As actively transcribed genes are generally associated with euchromatin, it is curious that topo IIβ is tightly associated with regions of heterochromatin where genes are poorly expressed. Furthermore, treatment of cells with histone deacetylase (HDAC) inhibitors results in the redistribution of topo IIβ from heterochromatic regions to euchromatic regions (Cowell et al., 2011). This important finding suggests two characteristics about topo IIβ. First, the enzyme is highly mobile within the context of a cell, and second, the localization of topo IIβ to heterochromatic regions may serve as an important mechanism by which the enzyme regulates gene expression. Interestingly, co-administration of HDAC inhibitors and topo II poisons in cultured cells has been found to induce a cytotoxic sensitization to topo II poison treatment, possibly coordinated with the redistribution of topo IIβ to more accessible regions of DNA (Marchion et al., 2005; Tsai et al., 2000).

Topo IIβ is important in regulating DNA topology in post-mitotic cells (including neurons, cardiomyocytes and spermatids), and also has established roles in replicating cells (Leduc et al., 2008; Nur-E-Kamal et al., 2007; Yang et al., 2000). For example, it has been demonstrated that topo IIβ association *in vivo* with Ku80 during the G_1 phase of the cell cycle is required to facilitate proper assembly of the pre-replicative complex (Rampakakis and Zannis-Hadjopoulos, 2009). Disruption of topo IIβ catalytic activity results in an extended G_1 phase (Rampakakis and Zannis-Hadjopoulos, 2009). Thus, a complete model of how topo IIβ functions in replicating cells with respect to the cell cycle remains lacking.

Like topo IIα, loss or disruption of topo IIβ activity leads to a loss of viability phenotype. In mouse models bearing homozygous knockouts of the topo IIβ isoform, mice develop *in utero*, but die shortly after birth due to a failure in the terminal innervation of skeletal muscle, leading to respiratory distress (Yang et al., 2000). Studies have postulated that this is due to the role topo IIβ plays in neurite outgrowth and growth cone formation, in addition to defects in cerebral stratification during corticogenesis (Lyu and Wang, 2003; Nur-E-Kamal et al., 2007). It is also of note that topo IIβ appears to play an important role in removing DNA supercoiling in elongating spermatids indicating another role of this isoform in development (Leduc et al., 2008).

The most striking role for topo IIβ is in the regulation of transcription due to its ability to generate transient double-stranded DNA breaks in the promoter regions of selected genes. Recent work has demonstrated that the isoform preferentially binds to sites demarcated by histone H3 lysine 4 methylation (Tiwari et al., 2012). These topo IIβ-mediated DNA breaks facilitate transcription by allowing local DNA relaxation, thus permitting access by transcriptional machinery (Ju et al., 2006). This restructuring occurs in association with the DNA-dependent protein kinase (DNA-PK), poly(ADP-ribose)

polymerase 1 (PARP-1), and appears to be most commonly associated with the regulation of hormone-regulated genes (Haffner et al., 2010; Hossain et al., 2009; Ju et al., 2006; Lin et al., 2009). Similar mechanisms involving topo IIβ activity may also be necessary for proper neuronal differentiation, as the regulation of several neuronal ion channels, receptors and transporters are strongly linked to topo IIβ catalytic activity (Sano et al., 2008; Tsutsui et al., 2001). More recently, the proper targeting of ganglion cell axons to their appropriate partners in the retina was found to dependent on topo IIβ-regulated transcription (Nevin et al., 2011). In addition, a role for topo IIβ (as well as topo I) has been identified in controlling the expression of long genes (those longer than 200 kbp) associated with autism spectrum disorder (King et al., 2013), suggesting that the role for topo IIβ in regulating transcription likely extends beyond the hormone-regulated genes initially described.

Therapeutic use of topo II-targeting chemotherapeutics, such as doxorubicin, etoposide and mitoxantrone, is associated with several significant long-term treatment-associated side effects. Genetic translocations have been associated with the development of secondary hematological malignancies (such as that between *MLL* and *RUNX1*), which can arise from topo IIβ poisoning (Cowell et al., 2012; Smith et al., 2014). The prevailing model is that these genes reside in close proximity within active transcription factories in which topo IIβ functions. Poisoning of the enzyme by treatment with drugs such as etoposide leads to chromosomal rearrangements within these factories. In this context, more diseases associated with deregulated transcriptional regulation or chromosomal rearrangements may also be associated with disruption of normal topo IIβ activity.

1.4.2 Topoisomerase IIα and its biological roles

The role of the topo IIα isoform has long been known to be important in actively

replicating cells. The expression of the enzyme fluctuates in a cell cycle-dependent manner. In cultured cell models, expression is reported to rise gradually throughout S-phase, reaching a peak three-fold increase in G_2/M. While this finding was postulated to be due to an increased requirement for chromosome segregation (Heck et al., 1988; Kimura et al., 1994; Woessner et al., 1991), later studies suggested that the increased amount of DNA present in dividing cells more strongly determines the level of expression (Isaacs et al., 1996; Stacey et al., 2000).

Mouse embryos lacking *top2a* (*top2a*$^{-/-}$) fail at the four to eight cell stage (Akimitsu et al., 2003). Down-regulation of *TOP2A* expression in mammalian cell models leads to mitotic catastrophe, due to a failure to decatenate sister chromatids (Akimitsu et al., 2003). In model organisms bearing only one topo II isoform (such as yeast), the absence of Top2 does not prevent replication from proceeding; however, newly replicated DNA becomes heavily intertwined, resulting in mitotic failure (Baxter and Diffley, 2008). In yeast models, the phenotype generated by the loss of Top2 differs from that in cells bearing a catalytically inactive Top2 mutant; cells expressing a catalytically inactive mutant are unable to complete replication at sites where two replication forks meet, leading to a stable G_2 arrest. A role for Top2 at sites of replication termination, which this suggests, is supported by the work of others (Fachinetti et al., 2010). It has been proposed that converging replication forks lead to an accumulation of positive supercoils, which must be properly relaxed for replication to finish.

Many difficult to replicate sites have been identified as fragile sites, and recent work identifies an essential role for yeast Top2 in mediating DNA breaks at these sites (Hashash et al., 2012). Indeed, sections of DNA that are prone to entanglement, such as telomeres and centromeres, require Top2 for proper separation (Germe et al., 2009;

Rouzeau et al., 2012). The inability for Top2 mutants to resolve DNA supercoiling may also impact chromatin structure on a higher level, such as with the spacing of nucleosomes, but not nucleosome incorporation (Germe and Hyrien, 2005), suggesting that supercoiling may provide a physical barrier to nucleosome deposition and require topo II activity to properly maintain chromosome structure.

Aside from its roles in relaxing DNA during replication, the requirement for topo IIα during cell cycle transition from the G_2 into mitosis has also been examined (Deming et al., 2001; Li et al., 2008; Luo et al., 2009). At this phase of the cell cycle, relaxation of supercoiled DNA substrates is of less concern, as cells have already completed DNA replication. Rather, the temporarily tetraploid (4N) cells must now ensure that each diploid (2N) complement of DNA is fully segregated, as the intertwining of sister chromatids poses a topological hurdle. During anaphase, the force generated by the mitotic spindles at the kinetochores is sufficient to generate DNA breaks, which could potentially lead to genetic aberrations such as aneuploidy and translocations.

Prior to entry into mitosis, cells begin the process of separating newly synthesized chromatids. Topo IIα and anaphase promoting complex (APC) have been shown *in vitro* to interact and stimulate topo IIα activity (Wang et al., 2008, 2010). When the interaction between APC and topo IIα is impaired in cell-based models, cells arrest at the G_2/M checkpoint and accumulate in the G_2 phase of the cell cycle. These cells display highly abnormal nuclear morphology, suggesting that this interaction has phenotypic consequences when disrupted. Indeed, this accumulation in G_2 manifests itself as a topo IIα-dependent checkpoint, the decatenation checkpoint, which can be functionally separated from the DNA-damage induced G_2/M checkpoint (Deming et al., 2001; Luo et al., 2009). Experiments have demonstrated that disruption of topo IIα, either through genetic

knockdown or pharmacological inhibition, activates this checkpoint, in which cells sense whether sister chromatids have been decatenated prior to DNA segregation (Bower et al., 2010; Luo et al., 2009). Upon checkpoint activation, mitosis is stalled until each pair of sister chromatids has been fully decatenated.

Activation of the decatenation checkpoint requires the interaction between topo IIα and the mediator of DNA damage checkpoint 1 (MDC1) protein, a protein with well-established roles in maintaining genomic stability. Catalytic inhibition of topo IIα with ICRF-193, which stalls sister chromatid separation, exposes a masked phosphorylation site (serine 1524) on topo IIα. Phosphorylation at this site promotes interaction with MDC1. MDC1 binding subsequently triggers activation of the decatenation checkpoint, halting progression through mitosis. A recent study has implicated a role for Plk1 in this process whereby a constitutively active Plk1 mutation resulted in the disruption of the interaction between MDC1 and topo IIα (Ando et al., 2013). Constitutively active Plk1 could override an ICRF-193-induced decatenation checkpoint suggesting that this Plk1 activity is essential upstream of the activation of this checkpoint. Thus, the role for topo IIα during mitosis is of utmost importance.

1.5 Pharmacological targeting of topoisomerase II by small molecule compounds

Due to the break-inducing nature of topo II, the enzyme has been a long-standing target of many drugs (Pommier, 2013; Pommier et al., 2010). Indeed, several early identified cancer chemotherapeutics that are still in clinical use today were determined to poison topo II many years after their initial discovery (Figure 1.5). The reason that pharmacological inhibition of topo II seems so appealing is two-fold. First, topo II is a ubiquitous enzyme that is essential for viability; no cell can undergo successful

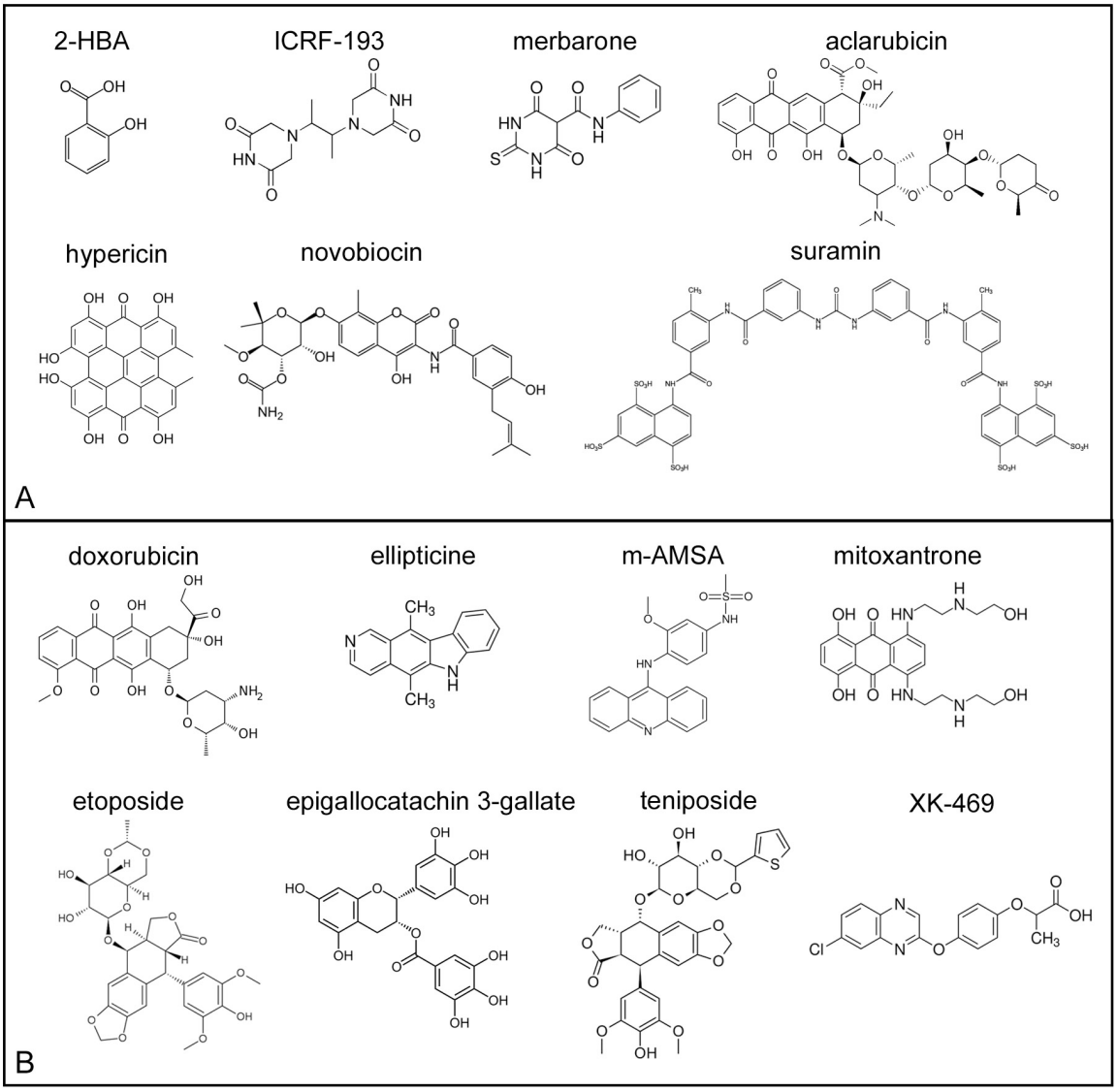
2-HBA
ICRF-193
merbarone
aclarubicin
hypericin
novobiocin
suramin
A
doxorubicin
ellipticine
m-AMSA
mitoxantrone
etoposide
epigallocatachin 3-gallate
teniposide
XK-469
B

Figure 1.5. Topoisomerase II targeting drugs.

Many compounds have been discovered to interfere with topo II activity and are classified as topoisomerase II poisons or catalytic inhibitors. (A) Catalytic inhibitors block topo II activity without generating DNA double-stranded breaks and do not trap the covalent cleavable complex. These compounds function through multiple mechanisms, including interfering with DNA binding (aclarubicin, suramin and hypericin), blocking DNA cleavage (merbarone and 2-HBA), and impeding ATP hydrolysis (novobiocin and 2-HBA). (B) Topo II poisons interfere with DNA religation and thus lead to the accumulation of DNA double-stranded breaks within the cell. These include the clinically used chemotherapeutics, doxorubicin, ellipticine, etoposide, mitoxantrone and teniposide. Other compounds have also been found to poison topo II, including epigallocatechin 3-gallate (a major substituent of green tea extracts) and XK-469, which has been found to be more selective towards the topo II beta isoform.

proliferation without its activity. Second, disruption of enzyme activity either results in the generation of DNA double-stranded breaks (highly cytotoxic lesions if not repaired appropriately), or results in an abrupt failure of cell division due to an inability to decatenate sister chromatids. While accumulation of strand breaks can result in programmed cell death, the latter causes cells to undergo mitotic catastrophe. Regardless, both outcomes are highly detrimental to cell viability.

The complexity of the catalytic cycle means that compounds targeting topo II do not necessarily block enzyme activity at the same step, nor do they necessarily bind to the same region of the enzyme. In the simplest form, drugs targeting topo II can be classified into two major classes: poisons and catalytic inhibitors (Larsen et al., 2003; Nitiss, 2009b). Poisons trap the enzyme as a covalent topo II-adduct on DNA, leading to the stabilization of DNA double-stranded breaks and turning the essential enzyme into a cellular poison. Removal of the adduct results in topo II degradation, thus the enzyme cannot re-enter the catalytic cycle (Mao et al., 2001). In contrast, catalytic inhibitors do not covalently trap the enzyme in a cleavable complex with DNA but block enzyme activity without stabilization of a DNA break. Catalytic inhibition does not require enzyme degradation and therefore the enzyme can potentially regain catalytic function should the inhibitor be removed.

Poisons, such as doxorubicin and etoposide (Table 1.3), trap the topo II-DNA complex after the formation of the covalent phosphotyrosyl bonds between the topo II homodimer and the DNA backbone (Wu et al., 2011a). This complex, referred to as the cleavable complex, prevents the enzyme from resealing the otherwise transient double-stranded DNA break. Furthermore, the covalent attachment means that the enzyme cannot dissociate from DNA. The considerable size of the trapped homodimer forms a topological barrier, preventing passage of the replication or transcription machinery through this site.

Table 1.3. Topoisomerase II poisons

Compound name	Drug class	Isoform specificity	Mechanism of action	References
Amsacrine	aminoacridine	alpha & beta	DNA intercalator; promotes topo II-DNA cleavage	Ketron et al., 2012
Doxorubicin	anthracycline	alpha & beta	cleavable complex stabilization; DNA intercalator; generates reactive oxygen species	Tewey et al., 1984a
Etoposide	Epipodophyllo-toxin	alpha & beta	cleavable complex stabilization; interferes with DNA religation	Wu et al., 2011a
Teniposide	Epipodophyllo-toxin	alpha & beta	cleavable complex stabilization	Chen et al., 1984
XK469	quinoxaline phenoxypro-pionic acid	beta	cleavable complex stabilization	Gao et al., 1999
NK314	benzo phenalthridine alkaloid	alpha	cleavable complex stabilization	Toyoda et al., 2008
Tricitinol B	citrinin derivatives	alpha & beta	blocks post-strand passage/religation equilibrium	Du et al., 2011
Mitoxantrone	anthracenedione	alpha & beta	cleavable complex stabilization; DNA intercalator	Ellis et al., 1990
Ellipticine	plant alkaloid	alpha & beta	cleavable complex stabilization	Tewey et al., 1984b
Epigallocate-chin gallate	catechin	alpha & beta	adducts topo II and prevents DNA ligation	Bandele and Osheroff, 2008

For this to be resolved, the enzyme must first be removed, exposing a double-stranded DNA break.

Previous work has demonstrated that the topo II adduct is removed in a proteasome-dependent manner (Mao et al., 2001; Zhang et al., 2006). Blockage of proteasome activity prevents cells from mounting a DNA double-stranded break response (Zhang et al., 2006) suggesting that removal of the enzyme is necessary to expose the DNA break. It was later postulated that the Bmi1/Ring1A E3 ubiquitin ligase is necessary for directing degradation of the topo II adduct (Alchanati et al., 2009). More recent work has also implicated MRE11 in removal of the protein-DNA adduct (Hartsuiker et al., 2009; Lee et al., 2012a). Degradation of the enzyme is imperfect, however, as the resulting DNA double-stranded break bears 5'-phosphotyrosyl end modifications. Thus, the canonical double-strand break repair pathways, such as non-homologous end joining and homologous recombination, cannot be engaged without first removing this end modification. To accomplish this, mammalian cells express a Mg^{2+}/Mn^{2+}-dependent phosphodiesterase, TDP2 (tyrosyl-DNA-phosphodiesterase 2), which removes this adduct, thereby converting the 5'-phosphotyrosyl linkage to a 5'-hydroxyl end (Cortes Ledesma et al., 2009). Loss of TDP2 is associated with increased sensitivity to topo II poisons, indicating its importance in repair of topo II adducts. Following removal of the 5'-phosphotyrosyl adduct, conventional DNA double-stranded break repair processes can be engaged to repair the damaged DNA.

1.5.2 Anthracyclines and the advent of doxorubicin

The majority of clinically used topo II poisons belong to the anthracycline or epipodophyllotoxin classes of drugs, although other classes, such as the anthracenediones also exist (Ellis et al., 1990). While epipodophyllotoxins, such as etoposide, were

discovered in the mid to late 1960s, anthracycline-based drugs can be traced back to the 1950s. Initially isolated by Italian scientists from the soil bacterium *Streptomyces peucetius*, daunomycin was found to have both antibiotic and anti-tumor properties, in addition to its distinct red pigment (Arcamone et al., 1969; Di Marco et al., 1964, 1969). At the same time, French researchers isolated the same compound, calling it rubidomycin (Dubost et al., 1964). Collectively, the groups agreed to change the name to daunorubicin. While in trials, daunorubicin was shown to be highly effective in treating various types of cancers, including leukemia and lymphoma. Unfortunately, its use was limited by treatment-related side effects, including severe cardiotoxicity (reviewed in Weiss, 1992). Subsequently, a second anti-tumor anthracycline, doxorubicin, was isolated. It remains one of the most widely used and clinically important anti-cancer chemotherapeutics.

The anthracycline-based chemotherapeutics were only found to target topo II some twenty years after their first discovery. Doxorubicin was first identified as a topo II poison when experiments revealed that a protein-DNA complex was formed after treatment with the drug (Tewey et al., 1984a). A similar observation was later made with etoposide (and its related epipodophyllotoxins). In early studies, it was noted that poisoning of the enzyme blocked religation of the cleaved DNA, resulting in an accumulation of DNA double-stranded breaks (Chen et al., 1984; Osheroff, 1989; Ross et al., 1984). Initial studies correlated the number of topo II-cleavable complexes formed in cells with relative sensitivity (Pommier et al., 1986).

Doxorubicin has been used for decades as a first-line chemotherapeutic for numerous cancer types, including breast and ovarian cancers, as well as leukemia, lymphoma, and sarcoma. The compound is toxic to cells through three different mechanisms. Aside from poisoning topo II, the planar structure of doxorubicin allows it to

intercalate DNA. In particular, the interaction with doxorubicin and DNA appears to occur in GC-rich regions (Manfait, Alix et al. 1982). Independently, the quinone moiety of doxorubicin can cycle through the iron-dependent Fenton reaction, generating reactive oxygen species (Goodman and Hochstein, 1977; Momparler et al., 1976; Pigram et al., 1972). In this reaction, doxorubicin is metabolized into its semiquinone by the action of xanthine oxidase or ferredoxin reductase. This semiquinone derivative then reacts with hydrogen peroxide and cellular iron to generate reactive oxygen species, while simultaneously processing the interconversion of Fe^{2+} to Fe^{3+}. Previous work has demonstrated that hydroxyl radical scavengers attenuate doxorubicin-induced DNA damage signaling (Kurz et al., 2004), suggesting that reactive oxygen species are important for the cytotoxic effects of doxorubicin. This finding corroborates earlier reports that antioxidants protect cells from doxorubicin-induced DNA damage (DeAtley et al., 1999). Together, these studies suggest that compounds with reactive oxygen sequestering properties could decrease the efficacy of doxorubicin.

1.6 Topoisomerase II catalytic inhibitors

Catalytic inhibitors block topo II activity at steps in the catalytic cycle that do not include stabilization of the cleavable complex (Fortune and Osheroff, 1998; Larsen et al., 2003). These include blocking DNA-enzyme interaction, preventing cleavage of DNA, interfering with ATP binding or hydrolysis, or trapping the enzyme as a closed clamp on DNA (Table 1.4). It is important to note that catalytic inhibitors do not necessarily block catalytic progression at a single step, but may concurrently inhibit multiple steps of the enzyme cycle. Regardless of their mechanism, the unifying hallmark of catalytic inhibitors

Table 1.4. Topoisomerase II catalytic inhibitors

Drug name	Drug classification	Isoform selectivity	Mechanism of action	References
Novobiocin	aminocoumarin	alpha & beta	inhibition of ATPase (GHKL domain) by acting as a competitive inhibitor of ATP	Miller et al., 1981
ICRF-193, ICRF-187, ICRF-154	bisdioxopipera-zines	alpha & beta	inhibition of ATPase activity (non-competitive); locks N-terminal protein clamp preventing release	Tanabe et al., 1991
Merbarone	thiobarbituate	alpha selective	non-covalent inhibition of DNA cleavage; does not intercalate DNA	Fortune and Osheroff, 1998
Aclarubicin	anthracycline	alpha & beta	intercalates DNA in the absence of topo II	Holm et al., 1994
Suramin	polysulfonated naphthylurea	unknown	prevents topo II binding similar to aclarubicin	Bojanski et al., 1992
AMPPNP	Non-hydroly-zable ATP analog	alpha & beta	inhibition of ATPase (GHKL domain)	Schmidt et al., 2012
Fostriecin	unknown	unknown	uncompetitive inhibitor of ATP hydrolysis	Boritzski et al., 1988
Hypericin	naphthodian-throne	unknown	inhibition of DNA binding	Peebles et al., 2001
Stauro-sporine	alkaloid	unknown	inhibits phosphodiester bond formation between tyrosine and DNA	Lassota et al., 1996

is that they do not stabilize the cleavable complex, and thus do not lead to the formation of topo II-mediated DNA breaks.

1.6.1 Inhibition of topoisomerase II-DNA interaction

As described earlier, the initial step of the topo II catalytic cycle requires that the enzyme bind to DNA in a sequence independent manner. Topo II recognizes the helical structure of DNA; however, compounds that distort the DNA structure, either by intercalating DNA or binding to the minor groove of the helix, can impair topo II binding.

DNA intercalators impair many biological processes, including DNA replication and transcription. These compounds generally contain planar, aromatic rings, which allow the compounds to insert between adjacent base pairs, thereby distorting the DNA helix (Armitage, 2005) . Some compounds bind the minor groove of DNA, wrapping around the outer face of the helix. These compounds generally are longer and flexible compared to intercalators.

As part of their interaction with DNA, intercalators decrease the twist and promote unwinding of the helix (Armitage, 2005; Ferguson and Denny, 2007). This unwinding promotes the formation of crossovers or supercoils (*i.e*., increase in writhe). Minor groove binders, meanwhile, also promote formation of DNA supercoils as a result of their binding and mask the DNA groove to prevent topo II binding.

Topo II catalytic inhibitors that block topo II-DNA interaction have been identified, although the specificity of these compounds is limited. Amsacrine, for example, can effectively intercalate DNA, thus blocking both DNA replication and transcription; however, it can also poison topo II (Wu et al., 2013). Recent work has suggested that the

reason amsacrine functions as an effective topo II poison is in part due to its ability to intercalate DNA (Ketron et al., 2012). Other compounds, such as hypericin, have also been identified as potent DNA intercalators by their ability to distort the DNA structure (Peebles et al., 2001).

1.6.2 Inhibition of DNA cleavage

Topo II cleaves DNA in an Mg^{2+}-dependent manner, where the Mg^{2+} ions are required for coordination of the cleavage transition state. Substitution with other divalent metal ions is associated with either a partial or complete loss of enzyme activity. The use of Ca^{2+} for example, allows for enzyme-mediated cleavage of DNA, but does not support DNA relaxation (Osheroff and Zechiedrich, 1987). This has proven useful in experimental settings for the study of religation *in vitro*, as reactions performed in the presence of Ca^{2+} result in kinetically competent topo II-DNA complexes that can be reversed by the addition of EDTA (Osheroff, 1987; Osheroff and Zechiedrich, 1987). In contrast, Cd^{2+} blocks topo II activity without generating cleavable complexes, suggesting that it acts as a catalytic inhibitor (Wu et al., 2011b). However, not all divalent metal cations act as topo II inhibitors. Cobalt (Co^{2+}) enhances topo II-mediated cleavage by decreasing DNA end ligation, classifying this cation as a poison, rather than an inhibitor (Baldwin et al., 2004).

Other compounds have been found to block topo II-mediated DNA cleavage as their primary mode of action. Merbarone, a thiobarbituric acid derivative, blocks DNA cleavage (Drake et al., 1989a; Fortune and Osheroff, 1998) and demonstrates modest selectivity for the alpha isoform (Drake et al., 1989b). Despite initial early success demonstrating antitumor effects in cell models, results from multiple clinical trials proved disappointing and further development of the drug was halted (Jones et al., 1993; Kraut et al., 1992;

Malik et al., 1997). Staurosporine has also been found to interfere with the topo II cleavage reaction (Du et al., 2011; Lassota et al., 1996). Staurosporine, which inhibits a wide range of protein kinases, blocks the formation of the phosphotyrosyl bond between topo II and DNA in an ATP-independent manner (Lassota et al., 1996).

1.6.3 Inhibition of ATPase activity

ATP is required as an energy cofactor for the topo II catalytic cycle. This cofactor is important for two steps of the catalytic cycle: strand passage and enzyme dissociation from DNA (Champoux, 2001; Nitiss, 2009a). The cyclical nature of the topo II catalytic cycle dictates that any compound that blocks enzyme activity should result in a concomitant decrease of ATP hydrolysis. Thus, whether the compound directly competes for the ATP binding site or not, loss of ATPase activity should be evident. Of all the sub-categories of catalytic inhibitors, those interfering with ATPase activity remain the largest group.

Compounds that directly impair topo II ATPase activity are classified into one of three categories, based on Michaelis-Menten kinetics: competitive, non-competitive and uncompetitive. Competitive inhibitors directly compete with ATP for the ATP-binding pocket of the enzyme, and the ability of the compound to inhibit topo II decreases as the concentration of the substrate (ATP) increases. A number of compounds that fall within this category structurally resemble ATP, including quinoline aminopurine 1 (QAP1) and adenosine 5'-(β,γ-imido)triphosphate (AMPPNP) (Chène et al., 2009; Larsen et al., 2003). While AMPPNP is used as a general non-hydrolyzable ATP analog for biochemical studies, novobiocin (and related aminocoumarins) has been used as an anti-bacterial drug as it also targets the ATPase domain of DNA gyrase. QAP1 was the first rationally designed

purine analog designed specifically to target human topo IIα and topo IIβ (Chène et al., 2009; Furet et al., 2009). Additional studies have used *in silico* molecular docking techniques to develop a series of catalytic inhibitors specific for the ATP-binding pocket (Baviskar et al., 2011; Huang et al., 2010), while others have been identified from natural sources, such as salvicine and gambogic acid (Hu et al., 2006; Qin et al., 2007).

Non-competitive inhibitors inhibit ATPase activity by blocking at a site distinct from the ATP binding site and, for the most part, have alternate mechanisms of action. In this regard, compounds that inhibit topo II ATPase activity non-competitively may, in fact, act on other steps of the catalytic cycle. For example, the bisdioxopiperazines block topo II activity by blocking the enzyme as a closed clamp; however, *in vitro* assays demonstrate loss of ATPase activity through a non-competitive mechanism (Morris et al., 2000). Similarly, vanadate, a commonly used protein phosphatase inhibitor, non-competitively blocks topo II ATPase activity, presumably by preventing the release of ADP from the binding pocket, while simultaneously promoting closed clamp formation (Harkins et al., 1998; Vaughn et al., 2005). The mechanism of vanadate is curious, however, as the compound acts at the ATP binding site but displays a non-competitive mode of inhibition. Regardless, non-competitive ATPase inhibitors provide a unique strategy to inhibiting the enzyme and understanding the binding site of the enzyme may lead to a better understanding of enzyme function.

Within the topo II literature, only one uncompetitive inhibitor, fostriecin, has been described (Boritzki et al., 1988). Uncompetitive inhibitors bind to the enzyme-substrate complex, unlike non-competitive inhibitors, which can bind to the enzyme at a site distinct from the substrate. Fostriecin displays potent anti-tumor effects *in vitro* and the compound was used in Phase I studies although with disappointing outcomes (Jong et al., 1999).

Subsequently, it was determined that the compound was more potent in targeting the protein phosphatases, PP1 and PP2A (Walsh et al., 1997), and its use as a topo II catalytic inhibitor diminished.

1.6.4 Stabilization of the closed clamp

Inhibitors that prevent topo II-mediated ATP hydrolysis and therefore do not allow the N-terminal clamp to open are referred to as closed clamp inhibitors. The best characterized of these are the bisdioxopiperazines (Classen et al., 2003; Germe and Hyrien, 2005; Morris et al., 2000).

The crystal structure of yeast Top2 in complex with ICRF-187 demonstrated that the compound locks the N-terminal protein clamp after ATP binding (Classen et al., 2003). This structure corroborates earlier findings that the bisdioxopiperazines block topo II turnover by preventing the enzyme from dissociating from DNA as well as studies on bisdioxopiperazine-resistant mutants with mutations in the N-terminal clamp (Patel et al., 2000). Experiments demonstrate that the topo II closed clamps trapped by ICRF-193 lead to the proteasomal degradation of the enzyme (Xiao et al., 2003). However, trapping of the enzyme in a closed clamp form results in the activation of the decatenation checkpoint at the G_2/M transition (Downes et al., 1994; Luo et al., 2009).

Treatment of cells with closed clamp inhibitors is cytotoxic. While increases in topo II-mediated DNA strand breaks do not occur, the absence of topo II activity during mitosis leads to cell death (Clarke et al., 1993; Khélifa and Beck, 1999). Curiously, yeast cells expressing human topo IIα and blocked in G_1 are also sensitive to bisdioxopiperazines, suggesting that interference with DNA processes during this phase are also critical to cell survival (Jensen et al., 2000).

Bisdioxopiperazines are not the only class of compounds that cause closed clamp formation. Recently, closed clamp inhibitors have been identified through proteomic profiling, in which researchers characterized proteome changes induced by almost three hundred compounds. Similarities in the changes of the proteome were used to identify a novel closed-clamp inhibitor, BNS-22, which displays properties similar to bisdioxopiperazines *in vitro* (Kawatani et al., 2011). Additionally, the benzene metabolite hydroquinone promotes closed clamps; however, it also interferes with other steps of the catalytic cycle, including DNA binding (Bender et al., 2006; Mondrala and Eastmond, 2010). The formation of closed clamps can also occur as a consequence of inhibition at other steps within the catalytic cycle. For example, as ATP-hydrolysis is required for enzyme dissociation, compounds that interfere with this process (as evidenced with non-hydrolysable ATP analogs), can increase closed clamp formation (Vaughn et al., 2005; Yang et al., 2009).

1.6.5 Other catalytic inhibitors

Many other catalytic inhibitors of topo II have been reported. In many cases, aside from demonstrating that topo II activity is inhibited, the experimental methods used have been incomplete and a specific mechanism for catalytic inhibition has not yet been determined. These include compounds such as suramin, sobuzoxane and 3,4-dihydroxybenzoic acid (Bojanowski et al., 1992; De Graff et al., 2003; Swift et al., 2008). As many of these compounds can target multiple enzymes, it is unlikely that these compounds could be used biochemically as topo II catalytic inhibitors (for example, in cell-based studies). What is clear from these studies is that many compounds that inhibit topo II are structurally related to previously characterized catalytic inhibitors. For example, the

anthocyanidin delphinidin was demonstrated to block both topo I and topo II activity without stabilizing topo II-DNA cleavable complexes (Esselen et al., 2009). This compound is closely related the topo II poison epigallocatechin gallate, but lacks a gallic acid moiety. Thus, structural similarities may provide clues as to how these compounds function.

1.7 Cellular responses to topoisomerase II targeting drugs

1.7.1 ATM-dependent DNA double-strand break repair

The ATM signaling pathway plays a critical role in mounting a coordinated cellular response to the topo II-mediated DNA strand break (Bhatti et al., 2011; Ciccia and Elledge, 2010). In particular, treatment of cells with topo II poisons activates the ATM (ataxia telangiectasia mutated) protein kinase, and its associated downstream effectors to signal the damage, arrest cell cycle progression and trigger DNA repair. ATM is so named as the absence or mutation of its gene (*ATM)* is associated with ataxia telangiectasia (A-T), a rare autosomal-recessive disorder characterized by progressive neurodegeneration, radiation sensitivity, immunodeficiency and a predisposition to cancer development (Bhatti et al., 2011; Shiloh, 1997).

ATM is a member of the phosphatidylinositol 3-kinase-like protein kinases (PIKKs). Among this family are two other members with significant roles in DNA repair: ATR (ataxia-telangiectasia and Rad3-related) and DNA-PKcs (DNA-dependent protein kinase catalytic subunit) (Durocher and Jackson, 2001). These proteins bear conserved domains, including a kinase domain that is flanked by the FAT (conserved in FRAP, ATM and TRRAP) and the FAT-C (FAT C-terminal) domains. While the kinase domain is

critical for the catalytic activity of the protein, the FAT and FAT-C domains are thought to be important for kinase regulation (Shiloh and Ziv, 2013).

In undamaged cells, ATM exists as a homodimer in an unphosphorylated state (Bhatti et al., 2011). Activation and recruitment of ATM to sites of DNA double-stranded breaks does not occur independently. In damaged cells, activation of ATM requires acetylation (on lysine 3016) by the Tip60 acetyltransferase and autophosphorylation (on serine 1981) *in trans*, leading to its dissociation into active monomers and recruitment to the break site. Recruitment of the active ATM monomer to DNA breaks also requires the function of the MRE11-Rad50-NBS1 (MRN) complex. While the role of NBS1 (Nijmegen breakage syndrome 1) is thought to be in mediating the interaction between ATM and the MRN complex, MRE11 and Rad50 play important roles in directly binding to DNA break ends. The initial recruitment of the MRN complex to break sites is independent of ATM; however, activation of the ATM-dependent signaling pathway requires the presence of the MRN complex. Interestingly, the recruitment of ATM to the MRN complex relies on the presence of Tip60, as the complex is important for directing Tip60 to a specific histone mark, histone H3 trimethylated on lysine 9 (H3K9me3). Thus, the interplay between multiple proteins is required for the successful activation and recruitment of ATM. Once recruited to the MRN complex, ATM phosphorylates all members of the complex, as well as a wide range of other effectors, including Chk2, SMC1 and p53, thus initiating a sequence of events to direct DNA repair, cell cycle arrest and if necessary, apoptosis. Experimentally, measuring phosphorylation states of these proteins using phosphospecific antisera provides a useful tool in determining cellular responses to double-strand break-inducing chemotherapeutics, such as the topo II poisons.

1.7.2 Chk2

The checkpoint kinase, Chk2, is a serine-threonine effector kinase and a direct phosphorylation substrate of ATM (Bartek et al., 2001; Stolz et al., 2011). Like ATM, Chk2 is primarily activated in response to DNA double-stranded breaks. In undamaged cells, Chk2 exists as an inactive monomer; upon damage, Chk2 localizes to sites of DNA breaks and becomes phosphorylated by ATM on threonine 68 (Antoni et al., 2007; Bartek et al., 2001). Previously, it has been demonstrated that Chk2 phosphorylation and kinase activation requires the upstream phosphorylation of NBS1 by ATM. The threonine 68 phosphorylation site of one Chk2 molecule is then recognized by a second Chk2 molecule, forming reciprocal molecular interactions in the forkhead-associated (FHA) domains of Chk2. The Chk2 homodimer subsequently autophosphorylates multiple sites *in trans* (serine 383 and 387). These sites reside in the activation loop of the Chk2 kinase, leading to full activation of the kinase. It should also be noted that the Chk2 protein contains multiple SQ/TQ PIKK phosphorylation sites within the extreme N-terminus of the enzyme, many of which play a regulatory role in enzyme function (Bartek et al., 2001).

As an effector, activated Chk2 does not remain at sites of DNA damage. After activation, the homodimer dissociates from the site of damage and phosphorylates multiple downstream substrates involved in cell cycle arrest (p53, Cdc25A and Cdc25C), DNA repair (BRCA1) and apoptosis (E2F1) (Stolz et al., 2011). Among the most critical pathways initiated by Chk2 are those involved in cell cycle checkpoints. Chk2 directly phosphorylates two important phosphatases, Cdc25A (on serine 123) and Cdc25C (on serine 216), which are involved in G_1- and G_2/M-arrest, respectively (Ahn and Prives, 2002; Falck et al., 2001). Chk2 also phosphorylates p53, a well-known mediator of cell cycle arrest and apoptosis, on serine 20 (Hirao et al., 2000). Phosphorylation on this site

destabilizes the interaction between p53 and its negative regulator MDM2; thus, Chk2 is involved in controlling p53 stabilization after DNA damage. Independently, Chk2 phosphorylates BRCA1 on serine 988, resulting in the activation of BRCA1 such that it can then promote DNA repair (Stolz et al., 2010). This interaction promotes DNA double-stranded break repair by homologous recombination, a repair pathway directly associated with ATM activity. Finally, multiple studies have identified a role of Chk2 in apoptosis through p53 (Jack et al., 2002) and E2F1; E2F-1 is phosphorylated after DNA damage by Chk2 (Stevens et al., 2003). Phosphorylation of E2F-1 transcriptionally activates p73 and Apaf-1, both of which have critical roles in cell cycle regulation and induction of apoptosis (Pommier et al., 2006).

1.7.3 SMC1

Cells also express a conserved protein complex, cohesin, necessary for maintaining chromosome structure (Lehmann, 2005; Watrin and Peters, 2006). The cohesin complex is comprised of two proteins belonging to the structural maintenance of chromosomes family: SMC1 and SMC3. Important structural features of these proteins include an N-terminal ATPase domain adjacent to a coiled-coil domain, which then connects to a C-terminal region. Both SMC1 and SMC3, in conjunction with two additional connector proteins, Scc1 and Scc3, form a protein clamp that allows the complex to bind newly formed sister chromatids.

The cohesin complex forms *de novo* at sites of DNA double-stranded breaks (Kim et al., 2002). At these breaks, members of the complex are phosphorylated by upstream kinases, such as ATM, to facilitate DNA repair. Specifically, ATM phosphorylates SMC1 at two sites, serines 957 and 966, after induction of DNA double-stranded breaks

(Matsuoka et al., 2007). Phosphorylation site mutants of SMC1 are defective in DNA repair and display radiosensitivity, highlighting the importance of this interaction. Moreover, inhibition of DNA replication after exposure to ionizing radiation (known as the intra-S-phase checkpoint) was compromised in cells expressing mutant SMC1 (Kitagawa et al., 2004).

1.7.4 p53

The tumor suppressor p53 has long been known to function in controlling aberrant cell growth through regulation of DNA repair, cell cycle arrest and apoptosis (Helton and Chen, 2007; Meek, 2004). Coined the 'guardian of the genome' (Lane, 1992), p53 expression is induced by many stimuli including DNA damage, interruption of DNA synthesis, hypoxic stress and mitotic inhibitors. Mutations in p53 are found in almost 50% of cancers, making it the most frequently mutated tumor suppressor. While the overwhelming majority (95%) of these mutations occur in the DNA-binding domain of p53, the remainder occurs within the oligomerization or transactivation domains of p53.

The transcriptional activity of p53 is tightly regulated in normal cells by the activity of MDM2, an ubiquitin ligase (Chène, 2003). This interaction maintains p53 at low basal levels in addition to localizing the protein to the cytoplasm. In response to DNA damage, ATM phosphorylates p53 on serine 15, disrupting its interaction with MDM2. This leads to its stabilization and translocation to the nucleus where the protein forms an active homotetramer. Through its DNA binding domain, p53 transactivates a number of genes associated with the cell stress response. Early findings discovered that p53 binds DNA in a sequence-specific manner, which led to the discovery of many p53 target genes, including those involved in cell cycle arrest, apoptosis and DNA repair (Meek, 2004). For example,

p53 modulates the expression of p21, leading to G_1 arrest, while regulation of 14-3-3σ expression modulates the G_2/M checkpoint. When the damage induced exceeds the cell's capacity for repair, p53 controls the expression of proteins involved in apoptosis, including members of the Bcl-2 family, Apaf-1 and proteins involved in TNF-mediated cell death. Considered together, it is clear that loss of p53 clearly promotes phenotypic changes associated with dysregulated cell growth.

1.8 Clinical implications of targeting topoisomerase II

1.8.1 Chemotherapeutic toxicities associated with topoisomerase II poisons

Drugs targeting topo II remain a mainstay of clinical oncology; however, their use is associated with a number of significant long-term toxicities, including the development of secondary malignancies and dose-limiting cardiomyopathy (Kollmannsberger et al., 1998; Relling et al., 1998; Yeh, 2006). Common topo II poisons, such as doxorubicin and etoposide, do not selectively target one isoform, nor can they be delivered exclusively to the tumor site as these drugs are administered systemically. The molecular mechanisms underlying these toxicities remained elusive for many years.

A 2007 study first proposed that inadvertent poisoning of the topo IIβ isoform may contribute to the observed deleterious secondary side effects (Azarova et al., 2007). In this work, mice bearing skin-specific deletions of the topo IIβ isoform were observed to be more prone to etoposide-induced melanomas than wild-type mice. Although melanoma is not commonly associated with etoposide administration, delayed onset leukemia is, often appearing years after initial exposure (Kollmannsberger et al., 1998). Early analysis of patients who developed these cancers found that a disproportionate number had

rearrangements in the MLL gene (Super et al., 1993); this was independent of pharmacokinetic clearance or drug metabolism (Pendleton et al., 2014; Relling et al., 1998). Follow-up from this work determined that the MLL gene contains strong consensus topo II-binding sequences, suggesting that the poisoning of topo II may provide a biochemical explanation behind the development of these malignancies (Le et al., 2009). Recent work has postulated that these rearrangements are due topo IIβ-induced DNA breaks, and that these breaks occur at active transcription factories in which topo IIβ is known to play a role (Cowell et al., 2012). Poisoning of topo IIβ within these transcription factories is hypothesized to stabilize a transcription-associated break, the repair of which leads to translocation within the MLL gene locus.

In addition to the increased risk of secondary malignancies, treatment with topo II poisons, in particular doxorubicin, is associated with severe dose-limiting cardiotoxicity (Yeh, 2006). Aside from its topo II-poisoning property, doxorubicin contains a quinone moiety allowing it to cycle through the iron-dependent Fenton reaction. It was long thought that this redox cycling was the primary factor driving doxorubicin-related cardiomyopathy as treatment with non-redox cycling topo II poisons, such as etoposide, does not trigger cardiovascular complications. Thus, synthesis of non-redox cycling derivatives of doxorubicin was pursued, leading to the development of 5-iminodaunorubicin (Lown et al., 1979; Tong et al., 1979). While this derivative showed an improved toxicity profile in laboratory studies, in patients 5-iminodaunorubicin was less well tolerated than doxorubicin and was subsequently abandoned (Weiss, 1992).

The role of reactive oxygen species in doxorubicin-mediated cardiotoxicity has been controversial. Many research efforts have and continue to implicate the use of reactive oxygen scavengers (antioxidants) as protective adjuvant therapies with doxorubicin

(Dayton et al., 2011; Quiles et al., 2002; Singal et al., 2000). Complicating matters, the clinically used cardioprotectant dexrazoxane, a bisdioxopiperazine used in conjunction with doxorubicin, is both a topo II catalytic inhibitor and an iron scavenger. While the ability of the compound to inhibit topo II is well known (as discussed earlier), its metabolite ADR-925 is a potent scavenger of free iron (Buss et al., 2004; Schroeder and Hasinoff, 2005; Yeh, 2006), thereby blocking the cycling of doxorubicin through the Fenton reaction.

Recent work has challenged the notion that the protective effects seen with dexrazoxane are attributable to its ability to sequester cellular iron and posits that the poisoning of topo IIβ is responsible for doxorubicin-mediated cardiotoxicity (Zhang et al., 2012). Using mouse models with cardiomyocyte-specific deletion of topo IIβ, animals treated with doxorubicin were protected from doxorubicin-induced cardiotoxicity. A role for topo IIβ should perhaps not be surprising as cardiomyocytes are post-mitotic and express very low levels of topo IIα. Clearly, increasing evidence supports the need for additional studies analyzing the contribution of the topo IIβ isoform to the long-term toxicities associated with topo II poisons, and investigations into the development of isoform-specific drugs as a therapeutic approach.

1.8.2 Clinical implementation of catalytic inhibitors in chemotherapy

The use of topo II catalytic inhibitors in clinical settings has been limited, owing largely to discouraging results from early clinical studies. Clinical trials with merbarone were disappointing, with no beneficial effect observed on tumor size or disease progression (Jones et al., 1993; Kraut et al., 1992; Malik et al., 1997). Later clinical trials utilizing dexrazoxane showed promising findings in reducing topo II poison-related cardiomyopathy and myelosuppression, without compromising the therapeutic effects of topo II poisons.

Preclinical studies using mouse models have demonstrated that dexrazoxane decreases myelosuppression associated with daunorubicin and etoposide treatment (Hofland et al., 2005a). Surprisingly, this protection does not occur in doxorubicin-treated mice. A separate study from the same group also demonstrated that co-treatment of patients with dexrazoxane in combination with etoposide and radiotherapy results in a synergistic cytotoxic effect leading to improved survival (Hofland et al., 2005b). Similar results with dexrazoxane have shown beneficial effects in reducing extravasation injuries associated with topo II poison treatment (Langer et al., 2012).

Although dexrazoxane was ineffective at reducing doxorubicin-associated myelosuppression, several landmark studies have found a beneficial use of dexrazoxane in preventing doxorubicin-induced cardiotoxicity both in adult and pediatric populations (Lipshultz et al., 2004, 2010; Swain et al., 1997; Testore et al., 2008). These studies assessed the ability of dexrazoxane to reduce cardiac injury in a population of children who received doxorubicin treatment for acute lymphoblastic leukemia, or in adults treated for breast cancer. Importantly, in addition to reducing the incidence of cardiotoxicity, these studies demonstrated that chemotherapeutic efficacy was not compromised in patients treated with dexrazoxane.

The use of dexrazoxane in children has not been without controversy (Walker et al., 2013). An initial study with dexrazoxane demonstrated a small but significant increased risk of developing secondary neoplasms (Tebbi et al., 2007); however, this risk was not reproduced in follow-up studies using larger pediatric patient populations (Barry et al., 2008; Vrooman et al., 2011). Despite this, the use of dexrazoxane in pediatric oncology remains limited.

One of the most intriguing and recent findings on the use of topo II inhibitors has been in relieving the repression of silenced genes. The loss of the maternal allele of the *Ube3a* ubiquitin ligase gene and concomitant epigenetic silencing of the intact paternal allele in neurons leads to Angelman syndrome, a neurodevelopmental disorder (Huang et al., 2011). Using a murine model of Angelman syndrome, it was observed that treatment with topo II catalytic inhibitors reversed the silencing of the paternal allele in several regions of the central nervous system, an effect that persisted after treatment was halted (Huang et al., 2011). These findings demonstrate that even transient impact on topo II catalytic activity can lead to persistent effects. It is clear that the therapeutic potential of topo II catalytic inhibitors remains an area requiring further study.

1.9 Salicylates

Salicylates have long been used for their anti-inflammatory, analgesic and anti-pyretic effects. The history and development of salicylates dates as far back as the 4th century BCE, when Assyrian physicians during the Sumerian period used extracts from willow leaves for their anti-inflammatory and pain-relieving properties (Jones, 2001; Mackowiak, 2000). It was centuries later that Hippocrates also prescribed the use of willow bark for alleviation of pain and inflammation (Rainsford, 2004). It was later determined that extracts from the willow bark contain significant levels of salicin, a β-glucoside alcohol that is metabolized to salicylic acid (2-hydroxybenzoic acid), resulting in a therapeutic effect (Figure 1.5). It was not until 1853 that French chemist Charles Frederic Gerhardt synthesized acetylsalicylic acid, a more stable form of salicylate. This was eventually patented and marketed to the general population by Friedrich Bayer & Co. in 1899 as Aspirin (Rainsford, 2004). Much has since been learned of the pharmaceutical

properties of salicylates (notably aspirin and its other derivatives) and their potential role in both preventive and interventional treatments.

Aspirin and its deacetylated primary metabolite share several common mechanisms of action and studies have demonstrated that their effects are widespread (Frantz and O'Neill, 1995), implying that salicylate is highly promiscuous in the cellular environment. Given the simple chemical structure of salicylate, it is perhaps not surprising that it has multiple targets.

The effects of salicylate on cell cycle progression and proliferation have been studied in detail. Salicylate stalls cell proliferation and induces apoptosis in both normal and malignant cells (Elder et al., 1996; Oh et al., 2003, 2003; Qiao et al., 1998; Stark et al., 2007); however, the mechanism underlying this is complex. Multiple pathways affected by salicylate treatment are involved in inflammation and apoptosis. Salicylate has well-studied effects on the nuclear factor kappa B (NFκB) pathway. Initial studies demonstrated that activation of NFκB by inflammatory cytokines such as tumor necrosis factor α (TNFα) was blocked by treatment with salicylate (Kopp and Ghosh, 1994; Pierce et al., 1996). In unstimulated cells, NFκB is sequestered in the cytoplasm by interaction with IκBα. In response to inflammatory stimuli, phosphorylation of IκBα by the IκB-kinase (IKK, a complex consisting of IKKα, IKKβ and IKKγ) leads to its degradation and release from NFκB. This allows NFκB to enter the nucleus where it acts as a transcription factor.

Initially, studies demonstrated that treatment with salicylate blocked degradation of IκBα associated with inflammatory stimuli (Kopp and Ghosh, 1994; Pierce et al., 1996). However, it was later determined that this was due to the ability of salicylate to block IKKβ activity by decreasing its association with ATP (Yin et al., 1998). Moreover, the p38 mitogen-activated protein kinase is activated by salicylate treatment, and appears to be

necessary for blocking TNFα-mediated IκBα degradation (Schwenger et al., 1998). In complementary studies, salicylate-mediated activation of NFκB has been shown to lead to caspase activation and apoptosis specifically in colorectal cancer cell lines, but not in cell lines derived from other tissue sites (Din et al., 2004; Stark et al., 2001, 2007). The reason for this discrimination remains undefined.

Recent work has demonstrated that salicylate treatment also results in the activation of AMP kinase (Hawley et al., 2012). AMP kinase plays an essential role in regulating energy levels dictated by intracellular concentrations of ATP. As cancer cells typically have alterations in energy balance, it is not entirely surprising that salicylate may have beneficial effects not only in normal tissue but malignancies as well. Indeed, this finding was corroborated in several colorectal cancer cell lines (Din et al., 2012), leaving the authors to postulate this as the reason why aspirin supplementation is most strongly associated with a decreased incidence in colorectal cancer.

Other signaling pathways, including those involved in p38 MAPK, Akt and ERK signaling are also modulated by salicylate (Derouet et al., 2006; Kim et al., 2008; Law et al., 2000; Schwenger et al., 1997, 1998). In addition, salicylate has also been noted to act as a histone deacetylase inhibitor (Di Renzo et al., 2008), which could impair many gene expression processes that rely on epigenetic control. Other studies further demonstrate the complexity of the NFκB signaling pathway through molecular linkages between p38, Akt and mTOR signaling (Baeza-Raja and Muñoz-Cánoves, 2004; Dan et al., 2008; Olson et al., 2007). This highlights and important possibility that salicylate action may also impinge on multiple pathways, outside of NFκB signaling, adding to its molecular complexity.

In contrast to the mechanisms conserved between acetylsalicylic acid and salicylic acid, the acetyl group of aspirin confers an additional mechanistic property. Aspirin is an

effective inhibitor of the cyclooxygenase family of enzymes (COX-1, COX-2 and COX-3); aspirin transfers its acetyl group to the catalytic serine of cyclooxygenase, irreversibly inhibiting its catalytic activity. Blocking cyclooxygenase inhibits the production of inflammatory mediators, including prostaglandins (specifically, PGE2). This spontaneous deacetylation generates salicylate (2-hydroxybenzoate), the primary metabolite of aspirin.

1.9.1 Salicylate-related compounds

Salicylates (including aspirin) are among the most widely used drugs worldwide, with an estimated 40,000 metric tonnes consumed annually (Warner and Mitchell, 2002). They are predominantly used in three ways: chronically in low doses for the prevention of secondary cardiovascular and cerebrovascular events, acutely in moderate doses for temporary relief of pain, fever or inflammation, or chronically in high doses as anti-inflammatories. In individuals taking 81 mg of aspirin per day, serum concentrations of approximately 25 μM have been measured (Blacklock et al., 2001; Paterson et al., 2006). In those on long-term, high dose therapy, serum concentrations reach the low millimolar range (Day et al., 1989; Hundal et al., 2002). In addition to aspirin, several clinically used anti-inflammatories are structural derivatives of salicylate. These include more potent drugs such as sulfasalazine, diflunisal, and salsalate, or those used in topical formulations, such as methyl salicylate.

Salicylic acid is a key plant hormone involved in the stress response and derivatives of salicylate (including the hydroxybenzoates) are widespread in plant-based diets. Studies have found that common spices, including cumin and turmeric, have salicylate concentrations upwards of 1% w/w (Paterson et al., 2006). This is of importance, particularly for those consuming a vegetarian diet and in whom plasma concentrations of

salicylates can vary between the high nanomolar to low micromolar range (Blacklock et al., 2001). Combined with the widespread use of salicylate-related medications, individuals may, unbeknownst to them, may be ingesting salicylates from a variety of unassuming sources. The effect of this remains to be investigated.

1.9.2 Salicylates and cancer

Given the availability and widespread use of salicylate-based drugs, robust epidemiological studies involving large populations can be carried out to examine whether exposure to salicylates impacts the morbidity or mortality of common diseases. For instance, consumption of low dose aspirin is associated with secondary vascular events in those with a history of myocardial infarction or stroke.

The strongest link has between cancer incidence and aspirin intake is with colorectal cancers. A considerable number of epidemiological studies, randomized control trials and meta-analyses have been conducted that, when considered together, strongly implicate a beneficial role for aspirin in reducing colorectal cancer incidence (Algra and Rothwell, 2012; Burn et al., 2011; Chan et al., 2008; Liao et al., 2012; Nishihara et al., 2013; Rothwell et al., 2011). Population-based studies have demonstrated reductions in cancer incidence from 25% to 40% depending on dose and duration of treatment. While it appears that low-dose aspirin is sufficient for a modest reduction in colorectal cancer incidence, higher doses show incrementally better reductions. However, benefits are typically not observed until at least three years of continual use (Rothwell et al., 2011).

Other cancers, including breast, Hodgkin's lymphoma, multiple myeloma and esophageal, also show a lower incidence in individuals taking aspirin (Birmann et al., 2014; Chang et al., 2011; Corley et al., 2003). While some of these studies have attributed these

effects to the anti-inflammatory property of aspirin, in esophageal cancers the correlation was only observed with aspirin users, but not those taking other non-steroidal anti-inflammatory drugs (Chang et al., 2011). The link between breast cancer risk and aspirin use is less well defined. Unlike the effects seen in colorectal cancer, most studies in which salicylate positively correlated with reduced breast cancer incidence were substantially smaller. Systematic reviews of the literature demonstrate risk reductions of 10-20% with aspirin therapy (Algra and Rothwell, 2012; Bosetti et al., 2012). While studies have also demonstrated a decreased risk of recurrence and cancer-related death in breast cancer patients taking aspirin (Chan et al., 2007; Holmes et al., 2010), these positive effects may be limited to those with long term (greater than 20 years) aspirin exposure (Chan et al., 2007).

Not all studies have reported a cancer risk reduction with aspirin. Two independent studies in Danish and Swedish populations found that low-dose aspirin or other NSAIDs provided no added protective effect for breast cancer incidence (Cronin-Fenton et al., 2010; Jonsson et al., 2013); however, one study noted that breast cancer patients were least likely to take aspirin compared to other cancer groups (Jonsson et al., 2013). Regardless of its potential merits on cancer risk reduction and cardiovascular health, regular intake of aspirin is not universally recommended due to the effects of the drug on platelet aggregation and irritation of the gastrointestinal tract (Derry and Loke, 2000; Weiss and Aledort, 1967).

1.9.3 The relationship between salicylate and doxorubicin

This thesis builds on previous work demonstrating that hydroxyl radical scavengers attenuate doxorubicin-induced DNA damage signaling (Kurz et al., 2004). Prior to the studies described herein, it was first asked if other hydroxyl radical scavengers (including

salicylate) might exert similar effects. Initial results demonstrated that salicylate effectively attenuated doxorubicin-induced DNA damage signaling in a variety of cell lines. However, this effect was not consistently observed with hydroxyl radical scavengers and DNA damage signaling induced by 5-iminodaunorubicin (a doxorubicin-analog incapable of redox cycling) was also blocked by salicylate. Thus, these early findings suggested that the effects of salicylate in attenuating doxorubicin-induced DNA damage signaling likely occurred through alternate mechanisms.

1.10 Hypothesis

Given the widespread use of aspirin and related salicylate-based drugs combined with the observation that salicylate attenuates doxorubicin-induced DNA damage signaling through a mechanism unlikely to be mediated by hydroxyl radicals, this thesis addresses the hypothesis that salicylate alters the cellular effects of topo II poisons, including doxorubicin and etoposide, by its actions as a catalytic inhibitor of topo II.

1.10.1 Specific aims

To address the stated hypothesis, the work described in this thesis was focused on three specific aims:

1) to determine the biological mechanism underlying salicylate-mediated attenuation of doxorubicin-induced DNA damage signaling in human breast cancer cells,
2) to determine the mechanism by which salicylate acts as a catalytic inhibitor of topo II,

3) and to examine whether compounds with structural similarities to salicylate act as catalytic inhibitors of topo II, with an aim to identifying key structural features modulating potency.

Chapter Two: Expanded Materials and Methods

While each research chapter contains its own Materials and Methods section (as part of the published manuscript), additional detail is provided here.

2.1 Human cell lines and tissue culture conditions

MCF-7 cells (ATCC HTB-22), a cell line derived from the pleural effusion of a woman with invasive ductal breast carcinoma, were cultured in Dulbecco's Modified Eagle's Media (DMEM) containing high glucose (Sigma-Aldrich, Oakville, ON) and supplemented with 10% [v/v] fetal bovine serum (FBS, Sigma-Aldrich), but without antibiotics. Cells were passaged 1:4 every 3 days, maintained at 37°C with 5% CO_2 and periodically verified to be mycoplasma-free (VenorGEM mycoplasma detection kit, Sigma-Aldrich).

2.2 Drug and irradiation treatment conditions

2.2.1 Preparation of drugs

Unless otherwise indicated, all compounds used for drug treatment were purchased from Sigma-Aldrich and stored at -20°C. Chemotherapeutics (doxorubicin, etoposide, camptothecin, topotecan, mitoxantrone, ICRF-193) were solubilized in dimethylsulfoxide (DMSO), protected from light and stored in aliquots at -20°C. Salicylate was prepared freshly to a stock concentration of 1 M prior to each use in 1X phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) and diluted to the indicated concentrations used in the experiements. Analogs and chemical derivatives of salicylate (3- and 4-hydroxybenzoates; 2,3-, 2,4-, 2,5-, 2,6-, 3,4- and 3,5-

dihydroxybenzoates; 2,3,4- and 3,4,5-trihydroxybenzoates; salicylamide, salicylaldehyde, 2-methylbenzoate and 2-fluorobenzoate) were dissolved in 20% DMSO to a stock concentrations of 100 mM. Anti-inflammatory drugs (acetylsalicylic acid (ASA), diflunisal, sulfasalazine, 5-aminosalicylic acid, and sulfapyridine) were prepared in 70% DMSO as 200 mM stocks. Ibuprofen was prepared freshly prior to each use in 1X PBS as 100 mM stock. Other non-steroidal anti-inflammatory drugs (NSAIDs) used in this study were prepared in 70% DMSO as 100 mM stocks.

2.2.2 Ionizing radiation

MCF-7 cells in culture medium were exposed to ionizing radiation using a Gamma-cell 1000 ^{137}Cs source (MDS Nordion, Ottawa, Canada) with a dose rate of 340 $cGy \cdot min^{-1}$.

2.3 Preparation of cell extracts

2.3.1 NET-N whole cell extraction

MCF-7 cells were seeded at 8 x 10^6 cells in 6 cm dishes approximately 36 h prior to addition of drug conditions, such that cells were logarithmically growing at the time of treatment. Immediately after treatment, cells were harvested and washed twice in cold 1X PBS, centrifuging at 800 x g for 5 min at 4°C after each wash. After the final wash, the PBS wash was removed and the cell pellet was resuspended in one packed cell volume of NET-N buffer (150 mM NaCl, 1 mM EDTA, 50 mM Tris-HCl pH 7.5, 1% [v/v] NP-40) containing both protease inhibitors (0.2 mM PMSF, 0.1 $\mu g\,mL^{-1}$ pepstatin A, 0.2 $\mu g\,mL^{-1}$ aprotinin, 0.2 $\mu g\,mL^{-1}$ leupeptin, dissolved in methanol) and phosphatase inhibitors (1 mM activated Na_3VO_4, 25 mM NaF). Cell suspensions were then incubated (10 min on ice) and

sonicated using a microtip (5 sec, 80% power, on ice). Extracts were centrifuged (12000 x g, 10 min, 4°C) and the cleared supernatant was transferred to a clean tube prior to protein quantification. Extracts were aliquoted, frozen rapidly in liquid nitrogen and stored at -80°C.

2.3.2 High salt nuclear extraction

MCF-7 cells were seeded at 8 x 10^6 cells in 6 cm dishes, 36 h prior to collection. Cells were harvested and washed twice in cold 1X PBS, centrifuging at 800 x g for 5 min at 4°C after each wash. After the final wash, the PBS wash was removed and the cell pellet was resuspended in ten pellet volumes of hypotonic lysis buffer (30 mM Tris-HCl pH 7.5, 3 mM $MgCl_2$, 10 mM KCl, 20% [v/v] glycerol) containing both protease and phosphatase inhibitors (described in section 2.3.1). Triton X-100 was then added to cell suspensions (final concentration 1% [v/v]) and the solution was gently mixed by inversion until a decrease in turbidity was observed. Samples were then incubated on ice for 10 minutes prior to centrifugation (12000 x g, 2 min, 4°C). After removal of the supernatant, the nuclear pellet was resuspended in 300 mM NaCl extraction buffer (30 mM Tris-HCl pH 7.5, 3 mM $MgCl_2$, 300 mM NaCl) containing protease inhibitors and incubated on ice for 30 min. The suspension was then centrifuged at 12000 x g, 2 min, 4°C and the supernatant containing the nuclear extract was retained for experimental use following protein quantification. Extracts were aliquoted, frozen rapidly in liquid nitrogen and stored at -80°C.

2.4 Immunoblotting

2.4.1 Immunoblotting of proteins smaller than 250 kDa

Protein quantification was performed using a Bio-Rad DC Protein assay kit (Hercules, CA) as per manufacturer's instructions using bovine serum albumin (BSA) as a standard. Absorbance readings were measured at 750 nm using a SpectraMax M2^e microplate reader (Molecular Devices, Sunnyvale, CA).

Protein samples (50 μg per well) were prepared in 1X Laemmli's buffer (0.083 M Tris pH 6.8, 2% [w/v] SDS, 10% [v/v] glycerol, 0.002% [w/v] bromophenol blue, 3.3% [v/v] 2-mercaptoethanol) and heated (5 min, 95°C) prior to loading. Proteins less than 250 kDa were resolved on a 10% acrylamide gel (9.6% [w/v] acrylamide, 0.4% [w/v] bis-acrylamide). Samples were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in running buffer (50 mM Tris, 384 mM glycine, 0.1% [w/v] SDS, pH 8.3). Following resolution, proteins were transferred onto nitrocellulose in transfer buffer (48 mM Tris-HCl, 39 mM glycine, 20% [v/v] methanol) at 100 V for 1 h. Membranes were then blocked in 25% [w/v] skim milk powder in 1X TBS-T (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% [v/v] Tween-20) for at least 1 h with gentle rocking. After washing (5 times 5 min each with 1X TBS-T), membranes were incubated with primary antibodies overnight according to antibody-specific conditions (Table 2.1). Following incubation with the primary antibody, membranes were washed with 1X TBS-T (5 times, 5 min each) prior to incubation with affinity-purified goat anti-mouse IgG or goat anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody (Bio-Rad; 1:3000 dilution in 1X TBS-T with 5% [w/v] skim milk powder; 1 h with gentle rocking). Immunoblots were subsequently washed 5 times with 1X TBS-T followed by a brief incubation with

Table 2.1. Antibodies used in immunoblotting studies

Antibody Target	Source	Catalog #	Host Animal	Working Dilution
Topo II alpha (K2)	Dr. David Kroll, Nature Research Center, Raleigh, NC	Custom	Rabbit polyclonal	1:1000
ATM pSer1981	Epitomics	2152-1	Rabbit monoclonal	1:5000
ATM (AM-9)	Millipore	05-513	Mouse monoclonal	1:500
p53 pSer15	Cell Signaling Technology	9284	Rabbit polyclonal	1:1000
p53 (DO-1)	Santa Cruz Biotechnology	SC-126	Mouse monoclonal	1:1000
SMC1 pSer957	Novus Biologicals	NB100-205	Rabbit polyclonal	1:1000
SMC1	Cell Signaling Technology	4802	Rabbit polyclonal	1:1000
Chk2 pThr68	Cell Signaling Technology	2661	Rabbit polyclonal	1:1000
Chk2	Cell Signaling Technology	2662	Rabbit polyclonal	1:1000
Actin	Sigma-Aldrich	A2066	Rabbit polyclonal	1:1000
I kappa B alpha pSer32	Cell Signaling Technology	14DA	Rabbit polyclonal	1:1000

enhanced chemiluminescence (ECL) reagent (0.1 M Tris-HCl pH 8.5, 12.5 mM luminol, 0.2 mM coumaric acid, 10% [v/v] H_2O_2) and exposure to film (SuperRx medical X-ray film, Fuji, Mississauga, ON).

2.4.2 Immunoblotting of proteins larger than 250 kDa

For proteins larger than 250 kDa, the following modifications were made during the immunoblotting procedure. Resolution of proteins by SDS-PAGE was carried out using an 8% low bis-acrylamide gel (7.9% [w/v] acrylamide, 0.1% [w/v] bis-acrylamide), but in the same running buffer and with the same stacking gel conditions. For these gels, proteins were transferred onto nitrocellulose in transfer buffer as described above, but containing 0.036% [w/v] SDS.

2.5 Comet Assay

Single cell gel electrophoresis (comet) assays under neutral conditions were utilized to assess DNA double-stranded breaks at the single cell level following treatment with doxorubicin agents. They were carried out using the CometAssay™ kit from Trevigen (Gaithersberg, MD) using a modified procedure. After treatment with the indicated drugs, MCF-7 cells were trypsinized, washed twice with cold 1X PBS and diluted to 1 x 10^5 cells mL^{-1}. This suspension was subsequently diluted 1:10 [v/v] in low melting point agarose and mounted onto pre-coated microscope slides and dried (25°C, overnight). The following day, slides were processed to examine comet tails under neutral conditions according to the manufacturer's protocol. Modifications to this protocol included extension of both the lysis and electrophoresis time to 45 min and 60 min, respectively. After processing, slides were stained with SYBR Green (diluted 1:10000 in 10 mM Tris-1 mM

EDTA (TE) buffer, pH 7.4) for 10 minutes at 4°C. Comet images were acquired using a Leica DMIRE2 microscope (Leica Microsystems, Richmond Hill, OH) and calculation of the comet length (head-to-tail distance) was measured using ImageJ (Schneider et al., 2012).

2.6 *In vivo* complex of enzyme (ICE) assay

The *in vivo* complex of enzyme (ICE) assay was carried out as previously described (Subramanian et al., 2001). Following treatment of cells, drug-containing medium was aspirated and cells were rapidly lysed with 1% [w/v] sarkosyl in 1X Tris-EDTA buffer (TE, 10 mM Tris-HCl pH 8.0, 1 mM EDTA) that had been equilibrated to 37°C. In polyallomer ultracentrifuge tubes (Beckman Coulter, Brea, CA), a CsCl step gradient consisting of 4 density layers (0.14 g mL^{-1}, 0.37 g mL^{-1}, 0.84 g mL^{-1}, 1.02 g mL^{-1}) was prepared. Lysates were layered on top of the CsCl density gradient and centrifuged (125,000 x g, 18 h, 20°C). The fractions containing the DNA were isolated from the column and sonicated. The DNA content of each fraction was quantified using a Nanodrop spectrophotometer (Thermo, Wilmington, DE). Samples were standardized such that equal amounts of DNA were blotted onto nitrocellulose membrane using a dot-blot apparatus (Bio-Rad). The membrane was subsequently immunoblotted for topo II (section 2.4.1).

2.7 Assessment of cell viability using Alamar Blue

To assess the cytotoxicity of chemotherapeutic treatments, MCF-7 cells were diluted to 4 x 10^4 cells mL^{-1} and seeded in flat-bottom 96-well plates, such that each well contained 4 x 10^3 cells. Plates were then incubated overnight at 37°C with 5% CO_2 to allow cells to adhere. Cells were then treated as outlined in the data chapters. Drug-containing

medium was aspirated and replaced with fresh medium following treatment and the incubation was continued at 37°C for an additional 96 h. After this period of time, Alamar Blue reagent (Invitrogen, Burlington, ON) was added to a final concentration of 5% [v/v] and plates were incubated for 90 min. The fluorescence reading from each well was then assessed ($\lambda_{ex/em}$: 570 nm/585 nm) using a SpectraMax M2^e microplate reader (Molecular Devices). Viability of drug-treated cells was assessed relative to that of untreated cells.

2.10 Electrophoretic mobility shift assay

2.10.1 Preparation of [α-^{32}P]-labelled binding site

A strong topo II binding site corresponding to residues 87-126 of the pBR322 plasmid as previously described (Kurz et al., 2000; Peebles et al., 2001) was synthesized as upper (5'-ATGAAATCTAACAATGCGCTCATCGTCAT-3') and lower (3'-TTTAG ATTGTTACGCGAGTAGCAGTAGGA-5') strands at the University of Calgary DNA Synthesis core facility.

The upper and lower strands (40 pmol each) were annealed in 5X Klenow buffer (250 mM Tris, pH 7.5, 50 mM $MgCl_2$) by heating to 95°C for 5 min prior to removal of the heat block and slow cooling to room temperature. Annealed oligonucleotides (5 pmol) were labeled in a fill-in reaction containing 40 μCi [α^{32}P] deoxycytidine triphosphate (dCTP, PerkinElmer, Waltham, MA) 0.1 mM dithiothreitol, 0.25 mM deoxynucleotide triphosphates (dNTPs, New England Biolabs, Ipswich, MA), and 10 U DNA polymerase I (Klenow fragment; New England Biolabs) for 20 min at room temperature. Following this incubation, dCTP (New England Biolabs) was added to a final concentration of 0.2 mM and the incubation was continued for an additional 10 min at room temperature. The

reaction was than purified using a Qiaquick Nucleotide Removal column (Qiagen, Valencia, CA) to separate the free nucleotides from the radiolabeled oligonucleotide. The radiolabeled oligonucleotide was further purified by phenol-chloroform extraction. Incorporation of the radioisotope was assessed using a scintillation counter (Beckman Coulter) and scintillant fluid (GE Healthcare, Little Chalfont, UK).

2.10.2 Electrophoretic mobility shift assay using nuclear extracts

High salt nuclear extracts were made from MCF-7 cells as section 2.3.2. Extracts (4 μg) were incubated with ~5-10 fmol of the radiolabeled binding site on ice for 30 min in reaction buffer (20 mM Tris, pH 7.6, 50 mM KCl, 1 mM EDTA, 2 mM $MgCl_2$, 10% [v/v] glycerol, 0.5 μg poly[dI:dC]). Prior to this, a 20 cm, 4% [w/v] acrylamide in 0.5X TBE (44.5 mM Tris, 44.5 mM sodium borate, 1 mM EDTA) gel was cast and allowed to polymerize overnight. The gel was electrophoresed at 100 V for 1 h in 0.5X TBE running buffer prior to loading of the samples. After changing the running buffer, the samples were loaded and reaction products resolved at 100 V for 2 h. Following resolution, the gel was transferred onto filter paper, dried under vacuum and exposed to film.

In experimental conditions where an unlabeled specific or non-specific competitor was used, increasing molar concentrations of the respective oligonucleotide was added. The specific and non-specific competitors were of similar nucleotide composition, and were annealed and blunt-ended as the labeled probe, with the exception of using non-radioactive dCTP.

Chapter Three: Sodium salicylate is a novel catalytic inhibitor of human DNA topoisomerase II alpha[1]

The foundation for this chapter began with the observation that N-acetyl cysteine, a hydroxyl radical scavenger, attenuates doxorubicin-induced DNA damage signaling (Kurz et al., 2004). This result suggested that the DNA-damaging effects of doxorubicin may be in part mediated through the generation of reactive oxygen species. To expand this observation, I examined whether other hydroxyl radical scavengers could exhibit similar effects, but surprisingly found this effect was not broadly observed among hydroxyl radical scavengers. Most interestingly, salicylate, a hydroxyl radical scavenger and anti-inflammatory with multiple mechanisms of action, was found to effectively attenuate doxorubicin-induced DNA damage signaling.

The following report describes these studies identifying a role for salicylate as a novel catalytic inhibitor of human topo IIα. The original publication has been reproduced in its entirety, but with the sections, figures and references reformatted to adhere to the guidelines outlined by the University of Calgary Faculty of Graduate Studies.

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[1] Bau JT and Kurz EU (2011) Sodium salicylate is a novel catalytic inhibitor of human DNA topoisomerase II alpha. Biochem. Pharmacol. 81:345-354.

3.1 Abstract

We have previously reported that pretreatment of human lymphoblastoid cells with the hydroxyl radical scavenger, N-acetyl cysteine, attenuates doxorubicin-induced DNA damage signalling through the ATM protein kinase. We sought to extend these studies to examine the effects of other hydroxyl radical scavengers in human breast cancer cells. Using MCF-7 cells, we observed that doxorubicin treatment triggered autophosphorylation of ATM on serine 1981 and the ATM-dependent activation of its downstream effectors p53, Chk2, and SMC1. Furthermore, we demonstrate that this effect was attenuated by pretreatment of cells with the hydroxyl radical scavengers sodium benzoate, sodium salicylate and, to a lesser extent, N-acetyl cysteine, but not Trolox™. Intriguingly, these effects were independent of doxorubicin's ability to redox cycle, were observed with multiple classes of topoisomerase II (E.C.5.99.1.3) poisons, but did not represent a general damage-attenuating response. In addition, the observed effects were independent of the ability of sodium salicylate to inhibit cyclooxygenase-2 or NFκB. We further demonstrate that sodium salicylate prevented doxorubicin-induced DNA double-strand break generation, which was attributable to inhibition of doxorubicin-stabilized topoisomerase IIα-DNA cleavable complex formation *in vivo*. Using topoisomerase IIα-DNA cleavage and decatenation assays, we determined that sodium salicylate is a catalytic inhibitor of topoisomerase IIα. Consistent with the observed inhibition of double-strand break formation, pretreatment of cells with sodium salicylate attenuated doxorubicin and etoposide cytotoxicity. These results demonstrate a novel mechanism of action for sodium salicylate and suggest that further study on the mechanism of topoisomerase II inhibition

and the effects of related therapeutics on doxorubicin and etoposide cytotoxicity are warranted.

3.2. Introduction

Since the emergence of nitrogen mustards and anti-metabolites in the 1940s, chemotherapy has played a key role in the treatment of both solid and haematological malignancies. Many of the most successful chemotherapeutics exert their cytotoxic effects as a consequence of inducing DNA damage that overwhelms a cell's capacity for repair. Among the most widely used of these DNA-damaging agents are those targeting topoisomerase IIα (topo II; E.C.5.99.1.3).

Topo II is an essential ATP-dependent enzyme that catalyzes the separation and unwinding of intertwined DNA strands for the processes of DNA replication and transcription, as well as in decatenating sister chromatids prior to mitosis (Burden and Osheroff, 1998; Nitiss, 2009a; Wang, 1996). It does so by generating a transient, covalently-coupled, double-stranded break (DSB) in DNA that permits a separate double-stranded DNA molecule to pass through the opening. It is this capacity for catalyzing the passage of a second, independent DNA molecule that allows for intertwined sister chromatids to be separated at mitosis and makes topo II indispensable for cell survival.

Beyond its critical physiological functions, topo II has emerged as the intracellular target of some of the most widely used anti-cancer chemotherapeutics, including doxorubicin (Adriamycin™) and etoposide (Vepesid™). These agents exert their cytotoxic effects by trapping topo II in covalent complexes with DNA, thereby stabilizing a normally transient intermediate in the enzyme's catalytic cycle (Burden and Osheroff, 1998; Nitiss, 2009b). As a result of this, these drugs 'poison' topo II and convert the enzyme into a

potent cellular toxin that induces DNA DSBs. In addition to inducing the accumulation of DNA DSBs through stabilization of topo II complexes on DNA, doxorubicin is also known to intercalate between DNA base pairs and to generate reactive oxygen species by virtue of the redox cycling of its quinone moiety through the Fenton reaction in the presence of cellular iron (Chabner et al., 2009; Nitiss, 2009b). It is these multiple effects that make doxorubicin one of the most widely used chemotherapeutics in clinical oncology.

Previously, while investigating the capacity for doxorubicin to induce DNA damage signalling through the ATM (ataxia telangiectasia mutated) protein kinase, we observed that pretreatment of human lymphoblastoid cells with N-acetyl cysteine (NAC), a hydroxyl radical scavenger, but not ascorbic acid, a superoxide scavenger, significantly attenuated the doxorubicin-mediated stimulation and phosphorylation of the ATM protein kinase and numerous downstream effectors, including p53, Chk2 and SMC1 (Kurz et al., 2004). These results suggested that hydroxyl radicals contribute to the doxorubicin-induced activation of ATM-dependent pathways. In this study, we sought to investigate whether this attenuation could be observed using other known hydroxyl radical scavengers and extend our investigations to breast cancer, as doxorubicin-based therapies are widely used in the treatment of both primary and metastatic breast tumors (Jensen, 2006).

Here, we describe studies demonstrating that sodium salicylate, the primary metabolite of aspirin, attenuates doxorubicin-induced DNA damage and DNA-damage signalling and that this effect is independent of doxorubicin's capacity to generate hydroxyl radicals. We have determined that this effect is also independent of sodium salicylate's capacity to inhibit cyclooxygenase-2 and NF-κB. We further report the novel and surprising finding that sodium salicylate attenuates the effects of doxorubicin as a consequence of its capacity to inhibit the topo II catalytic cycle without stabilization of the cleavable complex

and DSB formation and that it attenuates the cytotoxic effects of the topo II poisons doxorubicin and etoposide in cultured cells.

3.3. Materials and Methods

3.3.1 Reagents

Doxorubicin, etoposide, NAC, sodium benzoate, sodium salicylate, Trolox™ (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), mitoxantrone, diethyl maleate (DEM), camptothecin, topotecan, novobiocin and ICRF-193 were purchased from Sigma-Aldrich (Oakville, ON, Canada). 5-iminodaunorubicin (5-IDNR; NSC254681) was obtained through the National Cancer Institute/Developmental Therapeutics Program Open Chemical Repository (http://dtp.cancer.gov). Doxorubicin, mitoxantrone, etoposide, 5-IDNR, camptothecin, topotecan, and ICRF-193 stock solutions were prepared in DMSO (Sigma-Aldrich), protected from light and stored at -20°C. Novobiocin was prepared freshly in purified H_2O. NAC, sodium salicylate, and sodium benzoate were prepared freshly in 0.9% [w/v] NaCl prior to use in cell-based assays, with the pH of NAC adjusted to 7.5 with NaOH. Due to its limited solubility in aqueous buffer, Trolox™ was prepared freshly in DMSO prior to use. The ATM inhibitor (KU55933) was purchased from EMD Biosciences (San Diego, CA, USA), prepared as a stock solution in DMSO and stored at -80°C. DEM (97% purity), supplied as a liquid, was diluted freshly in DMSO prior to use. Rofecoxib, a generous gift from Dr. Aru Narendran (University of Calgary), was prepared in DMSO and stored at -20°C. Tumor necrosis factor alpha (TNFα) was a generous gift from Dr. Stephen Robbins (University of Calgary). All other chemicals and reagents

(unless otherwise noted) were of the highest quality available, nuclease free and purchased from either Sigma-Aldrich or EMD Chemicals (Gibbstown, NJ, USA).

3.3.2 Cell culture

Human breast cancer cells (MCF-7) were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Burlington, ON, Canada) supplemented with 10% fetal bovine serum (Sigma-Aldrich). Cells were maintained as logarithmic cultures at 37°C in a humidified atmosphere containing 5% CO_2. Treatments (chemotherapeutics and antioxidants) were added directly to the conditioned culture medium for the time indicated and at the concentration specified in each experiment. Chemotherapeutics prepared in DMSO were added to the culture medium with a minimum vehicle dilution of 1000-fold (maximum DMSO concentration 0.1%). Sodium salicylate prepared in 0.9% NaCl was added to the culture medium with a minimum vehicle dilution of 100-fold. In all experiments, untreated cells were exposed to volumes of drug vehicle equivalent to the volumes of added drug. Where indicated, cells were irradiated in the presence of serum-containing medium using a Gammacell 1000 cesium-137 source (MDS Nordion, Ottawa, Canada).

3.3.3 Antibodies

The p53-specific monoclonal antibody (DO-1, 1:2000) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). A phosphospecific antiserum to serine 15 of human p53 (1:3000) was purchased from Cell Signaling Technology (Beverly, MA, USA), as were the antibodies against SMC1 (1:1000), Chk2 phosphorylated at threonine 68 (1:1000) and Chk2 (1:1500). A phosphospecific antiserum to serine 1981 of ATM (1:5000)

was purchased from Epitomics (Burlingame, CA, USA), while the polyclonal antiserum to ATM (1:500) was purchased from Millipore (Billerica, MA, USA). A polyclonal antiserum against SMC1 phosphorylated at serine 957 (1:250) was purchased from Abcam (Cambridge, MA, USA) and a polyclonal antiserum specific for actin (1:1000) was purchased from Sigma-Aldrich. A phosphospecific antiserum to serine 32 of IκBα (1:1000) was purchased from Santa Cruz Biotechnology. A polyclonal rabbit antiserum against human DNA topo IIα (K2), raised to a fragment spanning amino acids 857-1448 of human topo II, was a generous gift from Dr. David Kroll (North Carolina Central University). Horseradish peroxidase-conjugated goat anti-mouse and goat anti-rabbit antibodies (1:3000) were purchased from Bio-Rad (Hercules, CA, USA).

3.3.4 Protein Extraction and Immunoblotting

Whole cell extracts were prepared from logarithmically growing MCF-7 cells as previously described (Kurz et al., 2004). Electrophoresis and immunoblotting conditions were as previously described (Kurz et al., 2004).

3.3.5 Image Analysis

Image analysis was performed using ImageQuant software (GE Healthcare Life Sciences, Baie d'Urfe, PQ, Canada). In the evaluation of specific phosphorylation events, phosphorylation levels were normalized to total protein levels by dividing the intensity of the phosphospecific signal by the intensity of the signal measured from blots using antibodies recognizing the total pool of protein.

3.3.6 Comet Assay

Single cell gel-electrophoresis (comet) assays were performed using the CometAssay® kit (Trevigen, Gaithersburg, MD, USA). Neutral comet assays were performed according to the manufacturer's protocol with the following modifications: post-treatment, cells were trypsinized, washed once with PBS and counted; 5 x 10^3 cells were used per sample. Slides were processed as outlined in the manufacturer's protocol, with the exception that slides were electrophoresed for 45 min at 20V (1V/cm) and at 4^{o}C. Comets were visualized using a Leica Microsystems DMRXA2 fluorescence microscope (Richmond Hill, ON, Canada). Measurement of tail moments was done using ImageJ (Rasband, 1997) or CometScore™ (www.autocomet.com; TriTek Corporation, Sumerduck, VA, USA). A minimum of fifty comets were measured for each experimental group. Tail moments across samples were analyzed by one-way ANOVA with a Tukey post-hoc test using Prism 4.0 software (GraphPad, La Jolla, CA, USA). $P < 0.01$ was considered significant.

3.3.7 In Vivo Complex of Enzyme Bioassay

Formation of stabilized topo II cleavable complexes was examined using the *in vivo* complex of enzyme (ICE) bioassay (Subramanian et al., 2001). In brief, following drug treatment MCF-7 cells were lysed rapidly in 1% w/v sarkosyl (OmniPur, EMD Chemicals). Lysates were then laid on a CsCl (OmniPur, EMD Chemicals) gradient and centrifuged at 125,000 x g for 18 h at 20°C. Fractions were collected, sonicated and DNA content of each fraction was determined spectrophotometrically using a Nanodrop spectrophotometer (Thermo, Wilmington, DE, USA). Fractions containing DNA were blotted onto

nitrocellulose membrane (Bio-Rad) using a dot-blot apparatus, followed by immunoblotting for topo II.

3.3.8 Topoisomerase II-mediated DNA Cleavage Assay

The effects sodium salicylate on topo II catalytic activity were analyzed based on a protocol modified from (Gantchev and Hunting, 1998) as previously described (Baker et al., 2001; Kurz et al., 2000). Purified topo II (8 units) (Topogen, Port Orange, FL, USA) was incubated in all experiments in a final reaction buffer containing 10 mM Tris pH 7.7, 50 mM NaCl, 50 mM KCl, 0.1 mM EDTA, 5 mM $MgCl_2$, 0.5 mM ATP, and 5 µg BSA (nuclease free; New England Biolabs, Ipswich, MA, USA) in the presence or absence of increasing concentrations of sodium salicylate (prepared in H_2O to avoid altering the NaCl concentration of the buffer) or vehicle (H_2O), and etoposide (or vehicle) on ice for ten min prior to initiating the reaction by the addition of 300 ng of pBR322 plasmid DNA (Promega, Madison, WI, USA). Reactions were incubated for exactly eight min at 37°C prior to the addition of stop buffer (10 mM EDTA, 200 mM NaCl), proteinase K (20 µg; Fermentas, Burlington, ON, Canada) treatment of samples and electrophoresis as described in (Baker et al., 2001; Kurz et al., 2000). To establish the effect of sodium salicylate co-incubation on etoposide-stabilized linear band formation, the relative intensity of the linear band (normalized to the intensity of the nicked circular band) in the presence of sodium salicylate was compared to that in the presence of etoposide alone.

3.3.9 Topoisomerase II-mediated kDNA decatenation assay

The direct effect of sodium salicylate on topo II catalytic activity was analyzed using a kinetoplast DNA (kDNA) assay and compared with known topo II catalytic

inhibitors. Purified topo II (2 units) (Topogen) was incubated in a reaction buffer containing 50 mM Tris pH 8.0, 150 mM NaCl, 10 mM $MgCl_2$, 0.5mM DTT, 2 mM ATP, and 30 µg/mL BSA in the presence or absence of increasing concentrations of sodium salicylate, novobiocin or ICRF-193 on ice for ten min prior to initiating the reaction by the addition of 180 ng of kDNA (Topogen). To account for any effects on catalytic activity, the final concentration of DMSO in all reactions was normalized to 0.3%. Reactions were incubated for exactly fifteen min at 37°C prior to the addition of stop buffer containing 1% sarkosyl, 5% glycerol and 0.025% bromophenol blue. Samples were electrophoresed through a 1% [w/v] agarose gel at 100V for 40 min and visualized with ethidium bromide.

3.3.10 Cytotoxicity assay

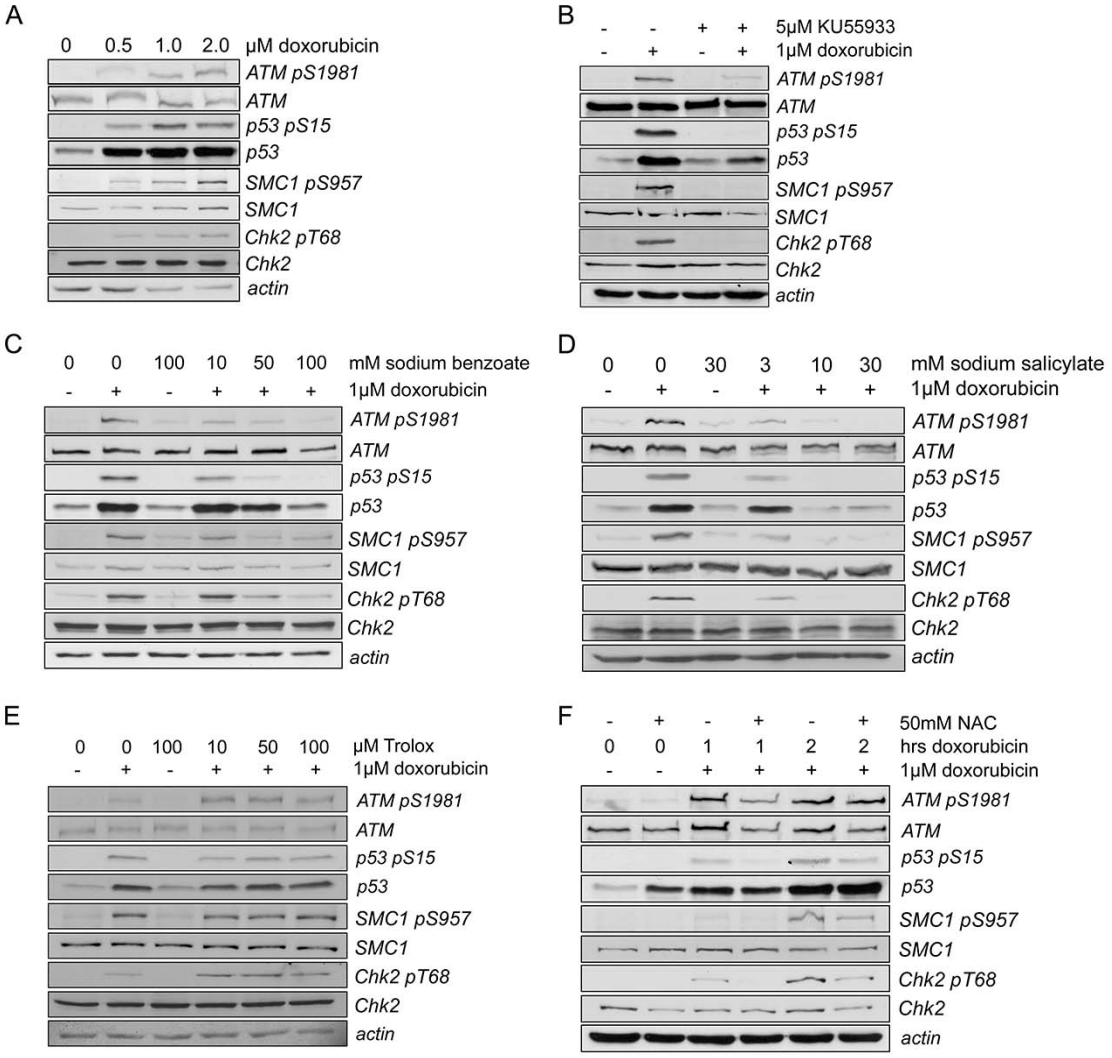
MCF-7 cells were seeded at a density of 5000 cells per well in 96-well plates for 16 h. In replicates of eight or twelve, cells were treated with increasing concentrations of sodium salicylate for 3 h, or were pretreated with sodium salicylate for 60 min prior to the addition of increasing concentrations of doxorubicin or etoposide and additional incubation for 2 h at 37°C. Subsequently, the cells were washed and resuspended in drug-free medium. After 96 h, the medium was removed and replaced with 100 µl Opti-MEM (Invitrogen) containing 5% [v/v] AlamarBlue™ (Invitrogen) and incubated for 3 h at 37°C. Fluorescence of AlamarBlue™ was measured at 585 nm following excitation at 570 nm using a SpectraMax M2E plate reader (Molecular Devices, Sunnyvale, CA, USA). Data are expressed as percent survival relative to untreated (vehicle only) or sodium salicylate treated cells, which were normalized to 100%. Survival of cells at a given dose of doxorubicin or etoposide in the presence or absence of pre-treatment with sodium salicylate

was analyzed by Student's t-test (df = 14 for each doxorubicin pair; df = 22 for each etoposide pair). $P < 0.01$ was considered statistically significant.

3.4. Results

3.4.1 Sodium salicylate attenuates doxorubicin-induced DNA-damage signalling through ATM in MCF-7 cells.

Based on our previous findings that the hydroxyl radical scavenger NAC was able to attenuate doxorubicin-induced DNA damage signalling in human lymphoblastoid cells in an ATM-dependent manner (Kurz et al., 2004), we sought to determine if similar effects were observed in ATM-proficient MCF-7 human breast cancer cells and whether other hydroxyl radical scavengers could mimic these effects. In a manner similar to that observed in human lymphoblastoid cells (Kurz et al., 2004), treatment of MCF-7 cells with doxorubicin led to the autophosphorylation of ATM (on serine 1981) and the phosphorylation of its downstream effectors p53 (on ser15), SMC1 (on ser957) and Chk2 (on thr68) in a dose- and time-dependent manner (Fig. 3.1A and data not shown). As observed with the human lymphoblastoid cells (Kurz et al., 2004), the doxorubicin-induced phosphorylation of these proteins is dependent on the ATM protein kinase as pretreatment of cells with a potent and specific inhibitor of ATM (KU55933) completely


A
0 0.5 1.0 2.0 μM doxorubicin
ATM pS1981
ATM
p53 pS15
p53
SMC1 pS957
SMC1
Chk2 pT68
Chk2
actin
B
- - + + 5μM KU55933
- + - + 1μM doxorubicin
ATM pS1981
ATM
p53 pS15
p53
SMC1 pS957
SMC1
Chk2 pT68
Chk2
actin
C
0 0 100 10 50 100 mM sodium benzoate
- + - + + + 1μM doxorubicin
ATM pS1981
ATM
p53 pS15
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SMC1 pS957
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Chk2 pT68
Chk2
actin
D
0 0 30 3 10 30 mM sodium salicylate
- + - + + + 1μM doxorubicin
ATM pS1981
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p53 pS15
p53
SMC1 pS957
SMC1
Chk2 pT68
Chk2
actin
E
0 0 100 10 50 100 μM Trolox
- + - + + + 1μM doxorubicin
ATM pS1981
ATM
p53 pS15
p53
SMC1 pS957
SMC1
Chk2 pT68
Chk2
actin
F
- + - + - + 50mM NAC
0 0 1 1 2 2 hrs doxorubicin
- - + + + + 1μM doxorubicin
ATM pS1981
ATM
p53 pS15
p53
SMC1 pS957
SMC1
Chk2 pT68
Chk2
actin

Figure 3.1. Doxorubicin-induced DNA damage signalling in MCF-7 cells is dependent on the ATM protein kinase and is attenuated by pretreatment of cells with NAC, sodium benzoate or sodium salicylate, but not Trolox™

(A) Human MCF-7 breast cancer cells were treated with increasing concentrations of doxorubicin for 2 h prior to the preparation of whole cell extracts and analysis by sequential immunoblotting with phosphospecific antisera against ATM (phosphorylated on Ser1981), p53 (phosphorylated on Ser15), SMC1 (phosphorylated on Ser957) and Chk2 (phosphorylated on Thr68), followed by immunoblotting for total pools of these proteins. Immunoblots were probed for actin as a loading control. (B) MCF-7 cells were either pretreated for 30 min with vehicle (-), or the ATM inhibitor Ku55933 (+; 5 μM) prior to treatment with doxorubicin (1 μM, 2 h) and immunoblotting as described in (A). (C - E) MCF-7 cells were either pretreated with vehicle (0) or increasing concentrations of sodium benzoate (C), sodium salicylate (D), or Trolox™ (E) for 1 h prior to the addition of doxorubicin (+; 1 μM) and further incubation for 2 h. Whole cell extracts were prepared and analyzed by immunoblotting as described in (A). (F) MCF-7 cells were either pretreated with vehicle (-) or NAC (+; 50 mM) for 30 min prior to the addition of doxorubicin (+; 1μM) and further incubation for 1 or 2 h. Whole cell extracts were prepared and analyzed by immunoblotting as described in (A). Representative immunoblots are shown for each compound from experiments performed independently at least three times.

abolished doxorubicin-induced phosphorylation of this kinase and its downstream effectors (Fig. 3.1B).

To investigate the effects of four known hydroxyl radical scavengers on this drug-induced signalling (Cervantes et al., 1988; Chernov and Stark, 1997; DeAtley et al., 1999), MCF-7 cells were pretreated with NAC, sodium benzoate, sodium salicylate or Trolox™, at concentrations previously shown in cultured cells to be effective, prior to the addition of doxorubicin. Pretreatment of cells with sodium benzoate (Fig. 3.1C) or sodium salicylate (Fig. 3.1D) completely attenuated doxorubicin-induced phosphorylation of ATM and its downstream effectors, while pretreatment with NAC led to partial attenuation of doxorubicin-induced signalling in MCF-7 cells, particularly evident at early time points of doxorubicin treatment (Fig. 3.1F). Although closely structurally related, the effects of sodium salicylate were consistently observed at a concentration at least one-fifth that required for sodium benzoate. Pretreatment of cells with Trolox™ had no effect on doxorubicin-induced activation of DNA damage signalling pathways (Fig. 3.1E).

3.4.2 Sodium salicylate attenuation of drug-induced DNA damage signalling is independent of doxorubicin's capacity to generate hydroxyl radicals.

Beyond its capacity to generate DNA DSBs through the poisoning of topo II, doxorubicin can intercalate DNA as well as generate hydroxyl radicals through the reaction of its quinone moiety with cytochrome P450 reductase and NADPH (Chabner et al., 2009). To evaluate whether doxorubicin-derived reactive oxygen species were required for the activation of ATM-dependent signalling and the observed attenuation by sodium salicylate, cell treatments were repeated using 5-iminodaunorubicin (5-IDNR). 5-IDNR is a doxorubicin analog with vastly attenuated redox cycling due to the absence of a quinone

ring (Johnston et al., 1983; Tong et al., 1979). Treatment of MCF-7 cells with 5-IDNR, at a dose (0.3 μM) equitoxic to that used for doxorubicin, robustly induced phosphorylation of ATM, Chk2, SMC1 and p53 (Figs. 3.2A and 3.S1A (supporting information)). Interestingly, as was observed for cells treated with doxorubicin, pretreatment of cells with sodium salicylate prior to the addition of 5-IDNR attenuated the phosphorylation of p53, Chk2 and SMC1, as well as modestly reducing the autophosphorylation of ATM on serine 1981. These data suggest that the DNA damage signalling through ATM observed following treatment of MCF-7 cells with doxorubicin is independent of doxorubicin's capacity to generate hydroxyl radicals. Similar attenuation of 5-IDNR signalling was observed in cells pretreated with sodium benzoate, and to a lesser extent NAC, but not with Trolox™ (data not shown).

3.4.3 Sodium salicylate attenuates DNA damage signalling from multiple classes of topoisomerase II poisons

To determine whether the observed effects of sodium salicylate were unique to the damage signalled from anthracycline-based topo II poisons, cell treatments were repeated using the topo II poisons mitoxantrone (an anthracenedione) or etoposide (an epipodophyllotoxin). Treatment of MCF-7 cells with mitoxantrone or etoposide, at doses (0.2 μM and 2.5 μM, respectively) equitoxic to that used for doxorubicin, robustly induced phosphorylation of ATM and its downstream effectors (Figs. 3.2B and 3.S1B). In a manner similar to that observed for doxorubicin, pre-treatment of cells with sodium salicylate attenuated the phosphorylation of all downstream effectors of ATM, with the

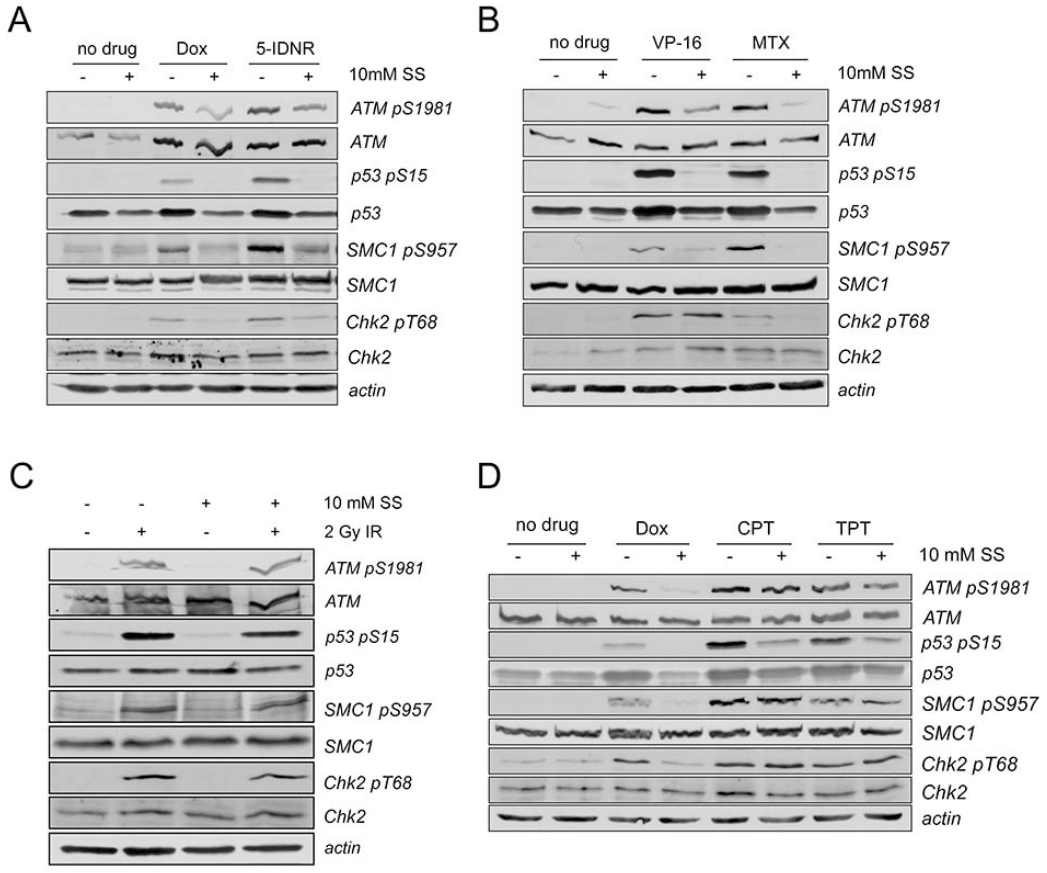

A
no drug
Dox
5-IDNR
10mM SS
ATM pS1981
ATM
p53 pS15
p53
SMC1 pS957
SMC1
Chk2 pT68
Chk2
actin
B
no drug
VP-16
MTX
10mM SS
ATM pS1981
ATM
p53 pS15
p53
SMC1 pS957
SMC1
Chk2 pT68
Chk2
actin
C
10 mM SS
2 Gy IR
ATM pS1981
ATM
p53 pS15
p53
SMC1 pS957
SMC1
Chk2 pT68
Chk2
actin
D
no drug
Dox
CPT
TPT
10 mM SS
ATM pS1981
ATM
p53 pS15
p53
SMC1 pS957
SMC1
Chk2 pT68
Chk2
actin

Figure 3.2. Attenuation of DNA damage signalling by sodium salicylate is independent of reactive oxygen species and is observed with multiple topo II poisons, but not IR or topoisomerase I poisons

(A) MCF-7 cells were pretreated (+) or not (-) with sodium salicylate (SS; 10 mM) for 1 h prior to the addition of doxorubicin (Dox; 1 μM), 5-iminodaunorubicin (5-IDNR; 0.3 μM) or no drug and continued incubation for 2 h prior to harvest. Whole cell extracts were prepared and analyzed by sequential immunoblotting for ATM and its downstream effectors as described in Fig. 3.1. Immunoblots were probed for actin as a loading control. (B) MCF-7 cells were pretreated with sodium salicylate as in (A) for 60 min prior to the addition of etoposide (VP-16; 2.5 μM) or mitoxantrone (MTX; 0.2 μM) and continued incubation for 2 h prior to harvest. Samples were harvested and analyzed as described in Fig. 3.1. (C) MCF-7 cells were pretreated with sodium salicylate as in (A) for 60 min prior to irradiation with 2 Gy and incubation for an additional 15 min prior to harvest. Samples were harvested and analyzed as described in Fig. 3.1. (D) MCF-7 cells were pretreated with sodium salicylate as in (A) for 60 min prior to the addition of doxorubicin (Dox; 1 μM), camptothecin (CPT; 10 μM), topotecan (TPT, 0.2 μM) or vehicle (no drug) and continued incubation for 2 h prior to harvest. Samples were harvested and analyzed as described in Fig. 3.1. Representative immunoblots are shown for each compound from experiments performed independently at least three times.

exception that sodium salicylate only modestly attenuated etoposide-stimulated phosphorylation of Chk2 on threonine 68. These data support the conclusion that the sodium salicylate-mediated attenuation of DNA damage signalling is not limited to anthracycline-based topo II poisons, but is more generally observed across multiple classes of topo II poisons.

3.4.4 Inhibition of DNA damage signalling by sodium salicylate is specific for topoisomerase II mediated damage.

It is well characterized that the ATM protein kinase is activated in response to DNA DSBs, triggering the phosphorylation and activation of its downstream effectors (Kurz and Lees-Miller, 2004; Lavin and Kozlov, 2007). To determine whether sodium salicylate is able to inhibit DNA damage signalling induced by DSB-inducing agents other than topo II poisons, MCF-7 cells were pretreated with sodium salicylate (10 mM, 1 hr) prior to exposure to ionizing radiation (IR) (2 Gy, 15 min recovery) (Figs. 3.2C and 3.S1). In contrast to the attenuation of DNA damage signalling observed in doxorubicin-treated cells, pretreatment of cells with sodium salicylate prior to irradiation failed to attenuate IR-induced phosphorylation of ATM and its downstream effectors, suggesting that sodium salicylate does not elicit a general cellular response to DNA double-stranded breaks (Figs. 3.2C and 3.S1C).

To determine whether the effects observed with sodium salicylate were specific to DNA damage induced by treatment of cells with topo II poisons, or a more general effect on topoisomerases in general, MCF-7 cells were pretreated with sodium salicylate (10 mM, 1 hr) prior to exposure to doxorubicin (1 μM, 2 hr) or the topoisomerase I poisons, camptothecin (10 μM, 2 hr) or topotecan (0.2 μM, 2 hr) (Figs 3.2D and 3.S1D). In contrast

to the attenuation of DNA damage signalling observed in doxorubicin-treated cells, pre-treatment of cells with sodium salicylate failed to attenuate camptothecin- or topotecan-induced phosphorylation of ATM and its downstream effectors (Figs 3.2D and 3.S1D).

3.4.5 Sodium salicylate attenuation of doxorubicin-induced DNA damage signalling is independent of effects on cyclooxygenase-2 and NFκB

Aside from its antioxidant properties, sodium salicylate is also known to inhibit cyclooxygenase-2 (COX-2) (Vane and Botting, 1998), as well as inhibiting the activation of nuclear factor kappa B (NFκB) through inhibition of IKKβ kinase, which normally phosphorylates the NFkB inhibitor IκBα to release active NFκB (Kopp and Ghosh, 1994; Yin et al., 1998). In contrast to this reported effect of sodium salicylate, pretreatment of MCF-7 cells with sodium salicylate inhibited TNFα-induced phosphorylation of IκBα by only 25% (Fig. 3.3A). In contrast, pretreatment of cells with diethyl maleate, another characterized inhibitor of NFκB (Horton et al., 1999; Lou and Kaplowitz, 2007), led to more robust (40%) inhibition of TNFα-induced phosphorylation of IκBα (Fig 3.3A). Although sodium salicylate was only a weak inhibitor of NFκB activation in our cells, we evaluated whether the inhibition of COX-2 or NFκB was responsible for the observed decrease in doxorubicin-induced DNA damage signalling following pretreatment with sodium salicylate by pretreating MCF-7 cells with rofecoxib (50μM), a COX-2 inhibitor (Hillson and Furst, 2000), or diethyl maleate (0.5mM) for 1 hour prior to the addition of doxorubicin (1μM, 2 h) (Fig. 3.3B,C). In contrast to sodium salicylate, neither rofecoxib nor diethyl maleate pretreatment of cells affected doxorubicin-induced DNA damage

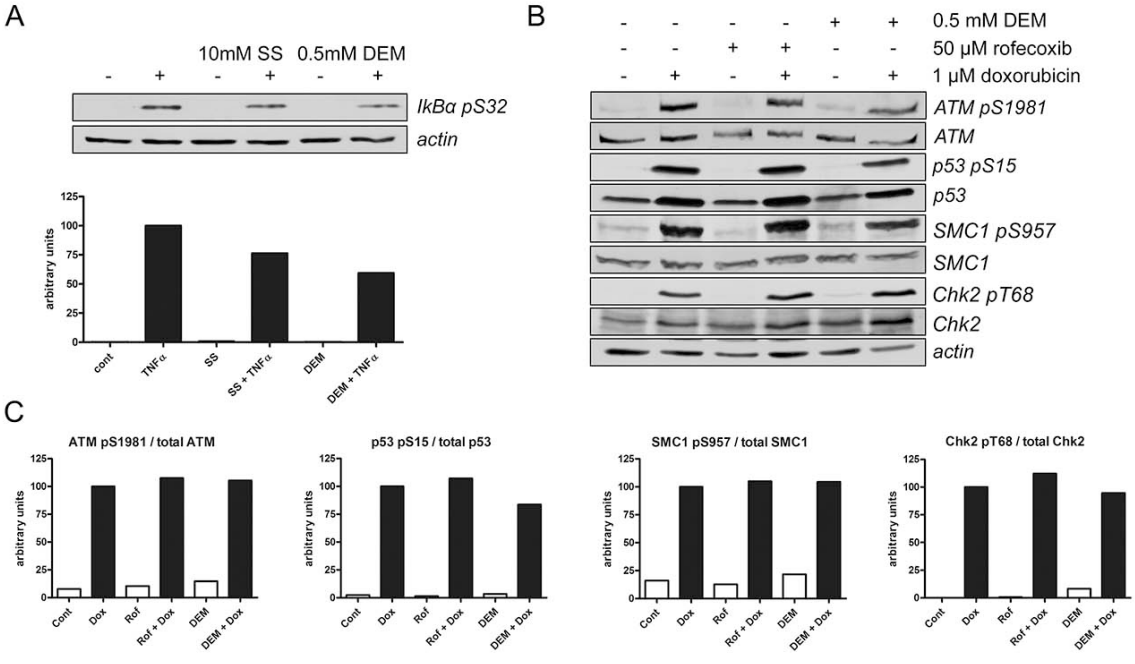

A
10mM SS 0.5mM DEM
- + - + - +
IkBα pS32
actin
arbitrary units
125
100
75
50
25
0
cont
TNFα
SS
SS + TNFα
DEM
DEM + TNFα
B
- - - - + + 0.5 mM DEM
- - + + - - 50 μM rofecoxib
- + - + - + 1 μM doxorubicin
ATM pS1981
ATM
p53 pS15
p53
SMC1 pS957
SMC1
Chk2 pT68
Chk2
actin
C
ATM pS1981 / total ATM
p53 pS15 / total p53
SMC1 pS957 / total SMC1
Chk2 pT68 / total Chk2
arbitrary units
Cont
Dox
Rof
Rof + Dox
DEM
DEM + Dox

Figure 3.3. Sodium salicylate attenuation of DNA damage signalling is not mediated by inhibition of cyclooxygenase-2 or NFκB

(A) MCF-7 cells were pretreated with sodium salicylate (SS; 10 mM) or diethyl maleate (DEM; 0.5 mM) for 1 h prior to stimulation with TNFα (20 ng/mL) and continued incubation for 5 min. Whole cell extracts were prepared and analyzed by immunoblotting using a phosphospecific antiserum to serine 32 of IκBα and actin as a loading control. A histogram showing the relative levels of phosphorylated IkBα (normalized to actin) as determined by densitometric analysis is shown. (B) MCF-7 cells were pretreated (+) or not (-) with rofecoxib (Rof; 50 μM) or diethyl maleate (DEM; 0.5 mM) for 1 h prior to the addition of doxorubicin (1 μM) and continued incubation for 2 h prior to harvest. Whole cell extracts were prepared and analyzed by sequential immunoblotting as described in Fig. 3.1. (C) The immunoblots shown in (B) were scanned and analyzed densitometrically. Phosphorylated protein levels were normalized to total levels of each respective protein analyzed. Data were then expressed relative to doxorubicin only-treated cells. Untreated cells or those only receiving pretreatment are represented by unfilled bars; cells treated with doxorubicin are represented by filled bars. Representative immunoblots are shown for each compound from experiments performed independently at least three times.

signalling, suggesting that the effects observed with sodium salicylate are independent of its capacity to inhibit COX-2 and NFκB.

3.4.6 Sodium salicylate attenuates doxorubicin-induced DNA DSB formation.

The absence of DNA damage signalling observed in cells pretreated with sodium salicylate could be attributed to one of two possibilities: (1) that salicylate impairs cellular signalling to and through the ATM protein kinase, or (2) that salicylate decreases or prevents the DNA damage induced by doxorubicin. Sodium salicylate did not alter IR-induced damage signalling through the ATM protein kinase (Fig. 3.2C), implying that sodium salicylate does not directly impair ATM function. To investigate the possibility that the decreased doxorubicin-induced DNA damage signalling observed with sodium salicylate pretreatment was the result of reduced DNA DSB formation, single cell gel electrophoresis (comet) assays under neutral conditions were carried out. As expected, cells treated with doxorubicin showed an accumulation of DNA DSBs (Fig. 3.4). However, when MCF-7 cells were incubated with sodium salicylate prior to doxorubicin treatment, DNA DSB formation was completely abrogated (Fig. 3.4). Together, these data demonstrate that pretreatment of cells with sodium salicylate prevents the induction of DNA DSBs by doxorubicin.

3.4.7 Sodium salicylate prevents doxorubicin-stabilized cleavable complex formation in vivo.

To evaluate whether the decreased levels of DNA strand breakage observed in the comet assay were the result of decreased cleavable complex stabilization by doxorubicin in the presence of sodium salicylate, an *in vivo* complex of enzyme (ICE) bioassay was

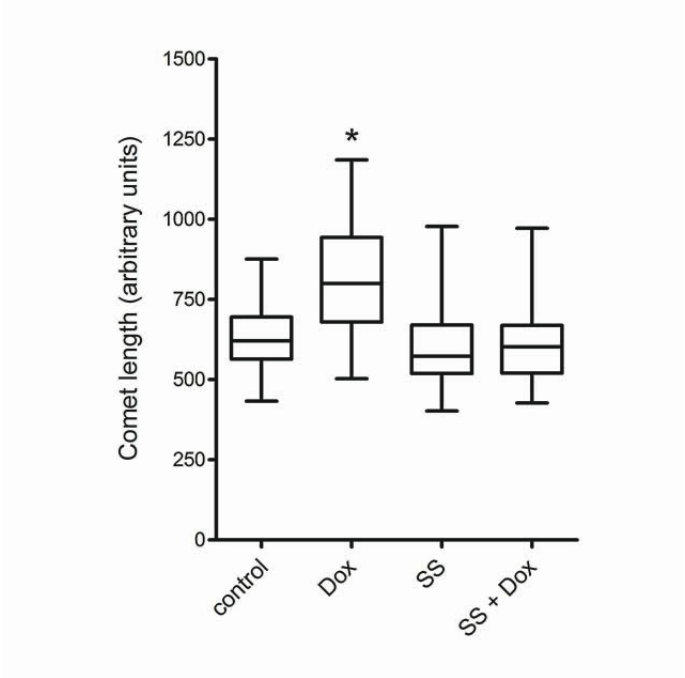


Figure 3.4. Sodium salicylate prevents the formation of doxorubicin-induced DNA DSBs. MCF-7 cells were pretreated or not with sodium salicylate (SS; 10 mM) for 1 h prior to the addition of doxorubicin (dox; 1 μM) or vehicle control (control; DMSO) and continued incubation for 2 h. Cells were then washed, embedded in agarose, lysed and electrophoresed under neutral conditions, and stained with SYBR green for analysis by the single cell gel electrophoresis (comet) assay. For each experimental group, a minimum of 50 comets was measured by fluorescence microscopy. Data are shown as box-whisker plots, with horizontal lines in each box representing the 25th, 50th and 75th percentiles and the vertical bars representing the minimum and maximum values measured for each experimental group. Data were analyzed by a one-way ANOVA with a Tukey post-hoc test. Comets from doxorubicin-treated cells differed significantly from all other conditions (*, $p < 0.0001$).

performed. By separating free protein from DNA-bound protein through a CsCl gradient, this assay allows for the quantitation of DNA-bound topo II after treatment of cells with a topo II poison. As expected, drug-stabilized topo II cleavable complexes were observed in cells treated with doxorubicin alone, but not those treated with ICRF-193, a catalytic inhibitor of topo II that does not stabilize topo II cleavable complexes (Fig. 3.5A). Treatment of cells with sodium salicylate alone did not lead to the accumulation of DNA-bound topo II above that observed in vehicle-treated cells. In contrast to cells treated with doxorubicin alone, no doxorubicin-stabilized cleavable complex formation was observed in cells pretreated with sodium salicylate (Fig. 3.5A), suggesting that sodium salicylate abrogates doxorubicin-stabilization of cleavable complexes, thereby preventing the formation of DSBs.

3.4.8 Sodium salicylate inhibits topoisomerase II catalytic function.

To assess the effects of sodium salicylate on topo II catalytic function, we used the modified cleavage assay of Gantchev and Hunting (Baker et al., 2001; Gantchev and Hunting, 1998; Kurz et al., 2000). This plasmid-based assay using purified topo II assesses catalytic activity by evaluating the relaxation of supercoiled DNA. Stabilization of topo II-DNA complexes, as achieved by topo II poisons, can be visualized through the appearance of a linear plasmid DNA band, migrating mid-way between the supercoiled and nicked circular species. Thus, this assay allows catalytic inhibition of topo II to be distinguished from cleavable complex stabilization by (1) the inhibition of DNA relaxation, and (2) the absence of linear band formation following incubation of the enzyme in the test compound. Incubation of topo II with increasing concentrations of sodium salicylate led to a dose-dependent inhibition of topo II catalytic activity as observed by the inhibition of DNA

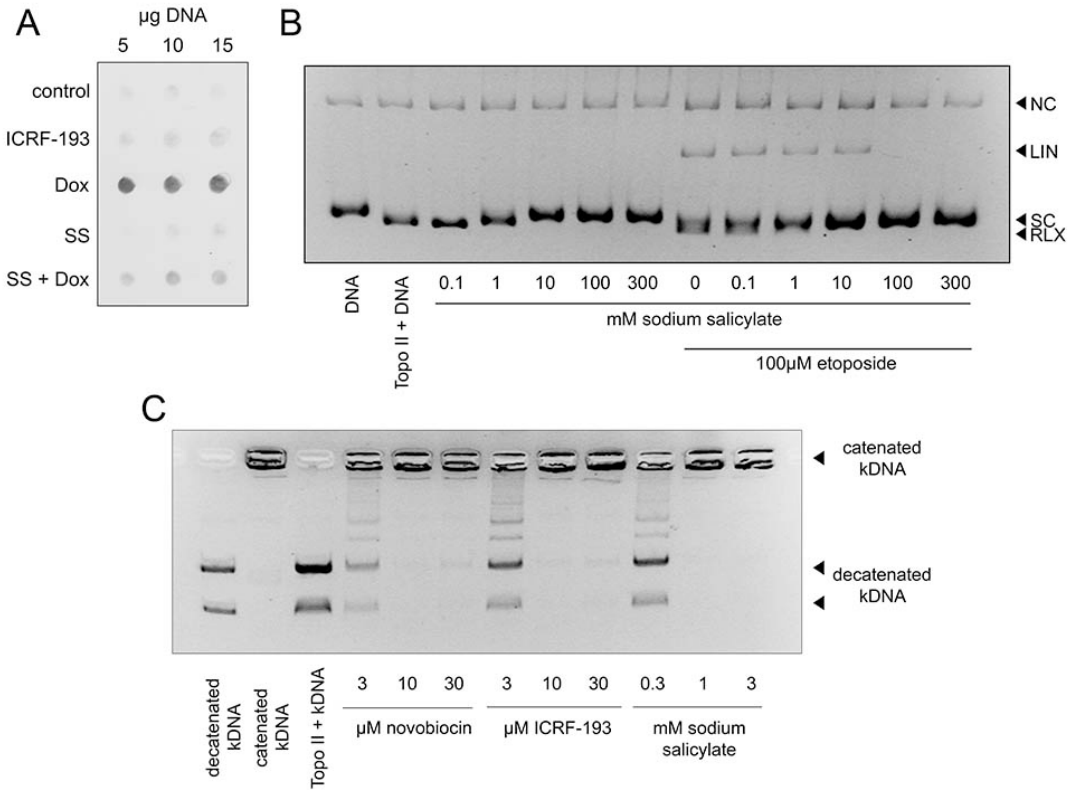

A
μg DNA
5
10
15
control
ICRF-193
Dox
SS
SS + Dox
B
NC
LIN
SC
RLX
DNA
Topo II + DNA
0.1
1
10
100
300
0
0.1
1
10
100
300
mM sodium salicylate
100μM etoposide
C
catenated kDNA
decatenated kDNA
decatenated kDNA
catenated kDNA
Topo II + kDNA
3
10
30
3
10
30
0.3
1
3
μM novobiocin
μM ICRF-193
mM sodium salicylate

Figure 3.5. Sodium salicylate attenuates doxorubicin-stabilized topoisomerase II-DNA cleavable complex formation and inhibits topoisomerase II catalytic activity

(A) MCF-7 cells were pretreated or not with sodium salicylate (SS; 10 mM, 1 h) prior to the addition of doxorubicin (1 μM) and continued incubation for an additional 2 h, or were treated with ICRF-193 (4 μM) for 2 h. Whole cell lysates were prepared, layered on CsCl gradients and centrifuged overnight to separate free proteins from those covalently bound to DNA. Gradient fractions were collected, sonicated and DNA concentrations were quantified spectrophotometrically. Increasing amounts of DNA (5, 10, or 15 μg) from each experimental condition were spotted onto nitrocellulose membrane using a dot-blot apparatus and analyzed for DNA-bound topo II by immunoblotting with a polyclonal antiserum to topo II. A representative immunoblot is shown. At least three independent replicate experiments were performed. (B) Purified topo II enzyme was preincubated in a buffer containing increasing concentrations of sodium salicylate in the presence or absence of etoposide (100 μM) for 10 min prior to the addition of supercoiled DNA. Reaction products were separated by agarose gel electrophoresis in the presence of ethidium bromide to allow for the separation of supercoiled (SC), relaxed closed-circular (RLX), and nicked circular (NC) DNA species. The experiment was completed at least three times with a representative experiment shown. (C) Purified topo II enzyme was preincubated in a buffer containing increasing concentrations of novobiocin (3, 10 or 30 μM), ICRF-193 (3, 10 or 30 μM) or sodium salicylate (0.3, 1 or 3 mM) for 10 min prior to the addition of kinetoplast DNA. Reaction products were separated by agarose gel electrophoresis followed by visualization with ethidium bromide. The experiment was completed at least three times with a representative gel shown.

relaxation (Fig. 3.5B). Consistent with the ICE bioassay, sodium salicylate did not stabilize topo II in a cleavable complex, as no linear band was observed, even at high salicylate concentrations (Fig. 3.5B). Consistent with its characterization as a topo II poison, incubation of topo II with etoposide resulted in the appearance of a linear DNA band as well as partial inhibition of DNA relaxation (Fig. 3.5B). In a manner similar to previously characterized topo II catalytic inhibitors (Tanabe et al., 1991), the addition of increasing concentrations of sodium salicylate in reactions containing etoposide led to both a dose-dependent inhibition of DNA relaxation and decreased formation of a linear DNA species (approximately 60% of control at 10 mM) (Fig. 3.5B).

As an alternative approach to examining the catalytic inhibition of topo II, sodium salicylate was compared to two known topo II inhibitors, novobiocin and ICRF-193, using a kDNA decatenation assay. While incubation of topo II with catenated kDNA led to complete decatenation of the substrate, preincubation with increasing concentrations of novobiocin, ICRF-193 or sodium salicylate led to dose-dependent inhibition of kDNA decatenation, further supporting the characterization of sodium salicylate as a catalytic inhibitor of topo II (Fig. 3.5C).

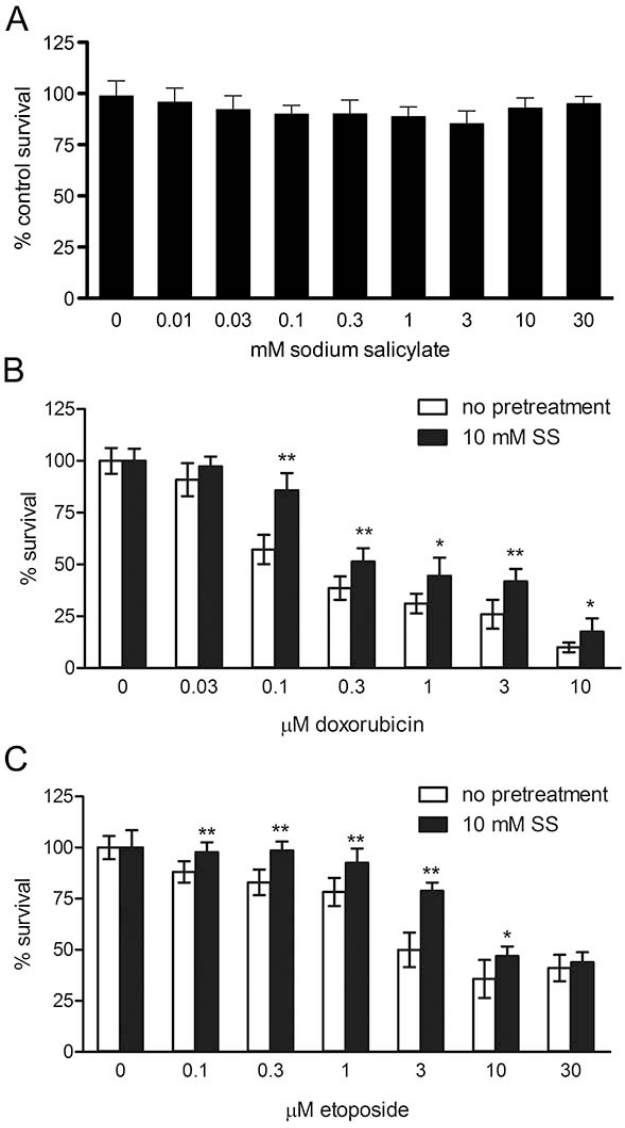
3.4.9 Sodium salicylate attenuates doxorubicin- and etoposide-induced cytotoxicity.

Given our demonstration that sodium salicylate inhibits DNA DSB induction by doxorubicin (Fig. 3.4) and decreases *in vivo* cleavable complex formation (Fig. 3.5A), we sought to determine if pretreatment of cells with sodium salicylate attenuated doxorubicin-induced cytotoxicity. We first analyzed the sensitivity of our cells to increasing concentrations of sodium salicylate using an AlamarBlue™-based cytotoxicity assay. In response to salicylate treatment alone, MCF-7 human breast cancer cells showed no

cytotoxicity at concentrations up to 30 mM (Fig 3.6A). To evaluate whether sodium salicylate affected doxorubicin cytotoxicity, MCF-7 cells were pretreated with sodium salicylate prior to the addition of a range of doxorubicin concentrations and subsequent analysis using AlamarBlue™ (Fig. 3.6B). Intriguingly, at clinically relevant concentrations of doxorubicin, sodium salicylate conferred a modest but significant cytoprotective effect, causing a shift in the IC_{50} of doxorubicin (Fig. 3.6B). This effect was not limited to doxorubicin, as pretreatment of cells with sodium salicylate also conferred protection from etoposide cytotoxicity (Fig. 3.6C).

3.5. Discussion

Topo II is an enzyme that is fundamental to multiple cellular processes and is absolutely required for the separation of sister chromatids prior to mitosis and, therefore, cell proliferation (Nitiss, 2009a). It is this absolute requirement for topo II that makes topo II poisons, such as doxorubicin, therapeutically effective in a broad range of tumor types. Through the stabilization of the topo II cleavable complex, treatment with topo II poisons causes the accumulation of DNA DSBs, which, if not repaired, lead to cell death. Hence, reduced DSB formation is associated with increased cell survival. Using several independent assays, we report here the novel finding that pretreatment of cells with sodium salicylate decreases doxorubicin-induced DNA DSB accumulation and doxorubicin-stabilized topo II-DNA cleavable complex formation through the direct inhibition of topo II catalytic activity. The consequence of this is demonstrated to be a reduction in the cytotoxicity of clinically relevant concentrations of doxorubicin and etoposide.


A
% control survival
125
100
75
50
25
0
0
0.01
0.03
0.1
0.3
1
3
10
30
mM sodium salicylate
B
no pretreatment
10 mM SS
% survival
**
**
*
**
*
0
0.03
0.1
0.3
1
3
10
μM doxorubicin
C
no pretreatment
10 mM SS
% survival
**
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*
0
0.1
0.3
1
3
10
30
μM etoposide

Figure 3.6. Sodium salicylate attenuates doxorubicin-induced cytotoxicity.

MCF-7 cells were seeded in 96-well plates at 5000 cells per well 16 h prior to drug treatment. (A) In replicates of eight, cells were treated with increasing concentrations of sodium salicylate for 3 h. Following treatment, the salicylate-containing medium was removed and replaced with drug-free medium. Incubations were continued for a further 96 h prior to the addition of AlamarBlue™ to determine viability. Data shown represent the mean and standard deviation of the percent cell survival normalized to cells not treated with sodium salicylate. (B) In replicates of eight, cells were treated (black bars) or not (white bars) with sodium salicylate (10 mM) for 1 h prior to the addition of increasing concentrations of doxorubicin and incubation for an additional 2 h. The medium was replaced and samples were handled as in (A). (C) In replicates of twelve, cells were treated (black bars) or not (white bars) with sodium salicylate (10 mM) for 1 h prior to the addition of increasing concentrations of etoposide and incubation for an additional 2 h. The medium was replaced and samples were handled as in (A). Data shown represent the mean and standard deviation of the percent cell survival normalized to cells not receiving doxorubicin or etoposide treatment. Data were analyzed by paired Student's t-tests (df=14 for doxorubicin; df=22 for etoposide). Statistical significance between samples at a given doxorubicin or etoposide concentration is denoted by asterisks (* $p<0.01$; ** $p<0.001$).

In earlier work using human lymphoblastoid cells, we observed an inhibition of doxorubicin-induced DNA damage signalling through the ATM protein kinase when cells were pretreated with NAC, a hydroxyl radical scavenger, but not ascorbic acid, a superoxide scavenger (Kurz et al., 2004). At the time, this finding was attributed to the scavenging of the hydroxyl radicals produced by doxorubicin in reaction with cytochrome P450 reductase, NADPH, and cellular iron through the Fenton reaction (Kurz et al., 2004). To follow-up on these observations, we set out to examine the effects of other known hydroxyl radical scavengers on the signalling of doxorubicin-induced DNA damage. The hydroxyl radical scavengers initially used in this study (sodium benzoate, sodium salicylate, NAC and Trolox™) were chosen as their scavenging properties have been investigated extensively (Cervantes et al., 1988; Chernov and Stark, 1997; DeAtley et al., 1999).

Sodium salicylate, at the concentrations used in this study, has been reported previously to inhibit the induction of p53 and its DNA-binding capacity in cells treated with doxorubicin (Chernov and Stark, 1997). It has also been shown to reduce the cytotoxicity of teniposide, an epipodophyllotoxin-related topo II poison (Fan et al., 2010). Using *in vitro* kinase assays, it was suggested that this was due to the direct inhibition of ATM and its related kinase, DNA-dependent protein kinase (DNA-PKcs) (Fan et al., 2010). However, our data demonstrating that sodium salicylate has no effect on IR- or topoisomerase I poison-induced DNA damage signalling would suggest that this is not the case *in vivo*. Sodium salicylate has also been reported to inhibit cell proliferation at low millimolar concentrations (Marra and Liao, 2001; Marra et al., 2000). Although we demonstrate here that low millimolar concentrations of sodium salicylate inhibit topo II

catalytic activity *in vitro*, inhibition of cell proliferation, at least following a short exposure to sodium salicylate, was not observed in our study.

Consistent with our finding that salicylate pretreatment decreases topo II-DNA interaction and this prevents the accumulation of DNA DSBs, pretreatment of normal human lymphocytes with acetylsalicylic acid (ASA) has been shown to decrease the appearance of chromosomal breaks induced by doxorubicin exposure (Antunes et al., 2007). While these authors suggest that this may be due to the capacity of ASA to scavenge free radicals generated by doxorubicin, their reported data are consistent with our findings using sodium salicylate that this could be a more direct effect of the inhibition of topo II. Similarly, it has been observed in a colorectal cell line that pretreatment of cells with low millimolar concentrations of ASA, but not a COX-2 selective inhibitor, decreases apoptosis and increases survival in response to etoposide administration (Ricchi et al., 2002).

Compounds that target topo II are classified into two categories: topo II poisons and topo II inhibitors (reviewed in (Larsen et al., 2003; Nitiss, 2009b)). Topo II poisons, such as doxorubicin and etoposide, stabilize the topo II-DNA covalent complex leading to an accumulation of DNA DSBs. In contrast, agents that act at other stages of the topo II catalytic cycle are termed topo II inhibitors. The catalytic inhibitors represent a diverse group of compounds that have been determined to inhibit topo II through numerous mechanisms. Among these are compounds that interfere with topo II-DNA binding (aclarubicin), inhibit ATP binding (novobiocin), prevent ATP hydrolysis (ICRF-193) or stabilize non-covalent topo II-DNA complexes in the absence of DNA scission (merbarone, ICRF-187). While it is clear from our data that sodium salicylate has characteristics

consistent with its identification as a topo II catalytic inhibitor, the precise mechanism by which it interferes with topo II catalytic function remains to be investigated.

It is unclear what chemical property of sodium salicylate contributes to its capacity to inhibit topo II. Our data with the non-redox cycling doxorubicin analog, 5-IDNR, as well as the non-anthracycline topo II poisons, mitoxantrone and etoposide, combined with the absence of an effect of pretreatment with Trolox™ would indicate that the observed effects are independent of hydroxyl radical formation by doxorubicin and radical scavenging by sodium salicylate. Additional support for this conclusion comes from the observation that pretreatment with sodium salicylate failed to dampen DNA damage signalling after treatment of cells with IR, which causes much of its DNA damage indirectly as a result of the ionization of cellular water leading to the production of hydroxyl and other radical species (Ward, 1988).

Sodium salicylate has also been extensively characterized as an inhibitor of NFκB activation (Kopp and Ghosh, 1994; Yin et al., 1998). Activation of NFκB is accompanied by the release from its inhibitory partner IκBα following IκBα phosphorylation (Nakanishi and Toi, 2005); thus, IκBα phosphorylation at serine 32 serves as a surrogate marker for monitoring NFκB activation. Although inhibition of IκBα phosphorylation has previously been reported in cells treated with sodium salicylate, treatment of MCF-7 cells with sodium salicylate in this study led to only a modest (less than 25%) inhibition of TNFα-stimulated IκBα phosphorylation. Despite the lack of NFκB inhibition by salicylate in these cells, sodium salicylate strongly attenuated doxorubicin-induced DNA damage signalling. In contrast, treatment of MCF-7 cells with DEM, which has also been characterized as an inhibitor of NFκB (Horton et al., 1999; Lou and Kaplowitz, 2007), led to greater than 40%

inhibition of IκBα phosphorylation yet did not alter doxorubicin-induced DNA damage signalling. Taken together, these data indicate that the changes observed in doxorubicin-induced DNA damage signalling with sodium salicylate are independent of any effects on NFκB.

Our results using sodium salicylate are provocative and illustrate the need for further study of salicylates and related therapeutics, including ASA. While this study focused on short exposure times and acute effects in cultured cells, additional experiments in cell and animal models using long-term, low or high dose exposure to salicylate or ASA are warranted. This is particularly necessary given that salicylate can reach millimolar concentrations in the plasma when used for both long-term anti-inflammatory/analgesic therapy in humans (Burke et al., 2009) and for short duration (3-7 days) treatments (van der Crabben et al., 2008; Koska et al., 2009), and can reach low millimolar concentrations in xenograft tumor sites in athymic mice following oral administration of a single low dose of ASA (Stark et al., 2007). It is tempting to speculate that partial inhibition of topo II catalytic activity by low doses of these agents may contribute to the anti-proliferative, cancer-chemopreventative nature of long-term salicylate-based therapies.

Beyond interest in chemoprevention, the data reported herein demonstrating that pretreatment of cells with sodium salicylate attenuates both doxorubicin and etoposide cytotoxicity, taken together with the published reports discussed, suggest that the anti-tumor efficacy of topo II-based therapies may be negatively affected by certain non-steroidal anti-inflammatory drugs (NSAIDs), particularly when the narrow therapeutic index of these anti-tumor chemotherapeutics is considered. Investigating a broader range of compounds and determining the precise mechanism by which salicylate inhibits topo II

catalytic activity may reveal additional evidence warranting the discouragement of NSAID co-administration in patients undergoing treatment for breast cancer or any of the broad-reaching malignancies using topo II poisons in their treatment regimens.

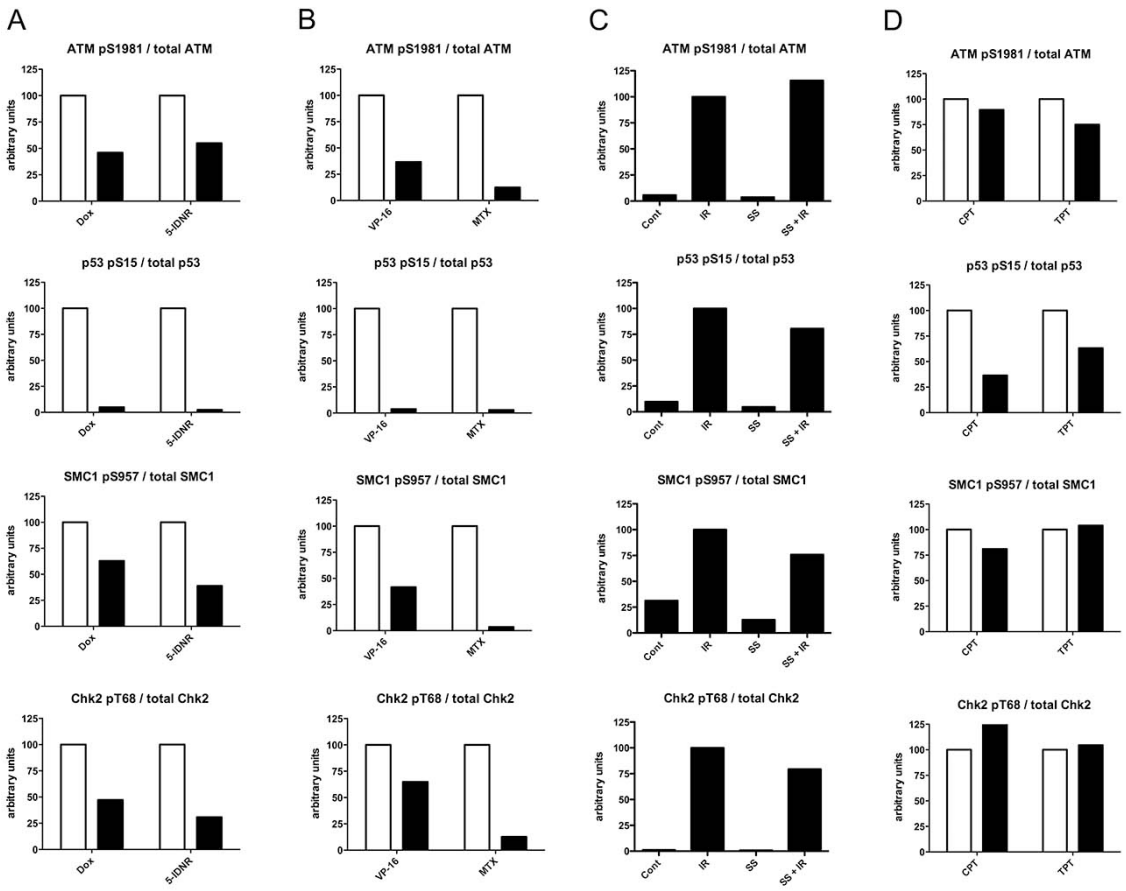

Figure 3.S1. Supplemental figure.

Attenuation of DNA damage signalling by sodium salicylate is independent of reactive oxygen species and is observed with multiple topo II poisons, but not IR or topoisomerase I poisons. Immunoblots shown in Fig. 3.2 were scanned and analyzed densitometrically as described in the Materials & Methods. Phosphorylated protein levels were normalized to the level of the total pool of the respective protein. Data are expressed relative to the respective DNA damaging agent (doxorubicin (Dox) or 5-IDNR in (A); etoposide (VP-16) or mitoxantrone (MTX) in (B); IR (C); camptothecin (CPT) or topotecan (TPT) in (D)).

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

In Chapter 3, I identified salicylate as a novel catalytic inhibitor of human topo IIα. As catalytic inhibitors can impair enzyme activity at a number of steps within the catalytic cycle, I set out to determine the biochemical mechanism by which salicylate inhibits topo II catalytic activity. Our studies determined that salicylate impairs topo II-mediated DNA cleavage and as a result leads to a non-competitive decrease in ATP hydrolysis. During our findings, I also noted that topo II inhibition by salicylate was selective for the topo IIα isoform.

The complete findings are contained in the following report[1]. The original publication has been reproduced in its entirety, but with the sections, figures and references reformatted to adhere to the guidelines outlined by the University of Calgary Faculty of Graduate Studies.

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[1] Bau JT, Kang Z, Austin CA, and Kurz EU (2014) Salicylate, a catalytic inhibitor of topoisomerase II, inhibits DNA cleavage and is selective for the alpha isoform. Mol. Pharmacol. 85:198-207.

4.1 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.

4.2 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.

4.3 Materials and Methods

4.3.1 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 H_2O and stored at -20°C. Salicylate was prepared freshly in distilled H_2O 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).

4.3.2 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% CO_2. 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 Na_2HPO_4, 1.8 mM KH_2PO_4) and resuspended in hypotonic lysis buffer (30 mM Tris pH 7.5, 3 mM $MgCl_2$, 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 12,000*g* 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 $MgCl_2$, 300 mM NaCl) containing protease inhibitors. Following an additional 30 min incubation on ice, the suspension was centrifuged at 12,000 x g 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.

4.3.3 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 $MgCl_2$, 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.

4.3.4 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 NaH_2PO_4) overnight at 10 V. DNA bands were visualized after staining with 1 μg mL^{-1} ethidium bromide.

4.3.5 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-^{32}P]-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 $MgCl_2$, 10% v/v glycerol, 0.5 μg poly[dI: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.

4.3.6 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 $MgCl_2$, 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 50*g* 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.

4.3.7 ATP hydrolysis assay using [γ^{32}P]-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-^{32}P]-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) polyethyleneimine-impregnated cellulose plates (Sigma-Aldrich) and air-dried. Reaction products were resolved by developing

plates with freshly prepared 400 mM NH_4HCO_3. 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.

4.3.8 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.

4.3.9 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 $CaCl_2$, 0.03 μg μl^{-1} bovine serum albumin. Reactions were incubated at 37°C for 15 min prior 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.

4.3.10 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.

4.3.11 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 $CaCl_2$, 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 $MgCl_2$ and transfered 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.

4.3.12 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.

4.4 Results

4.4.1 Salicylate does not intercalate DNA and does not interfere with topoisomerase 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. 4.1A). In contrast, the addition of increasing

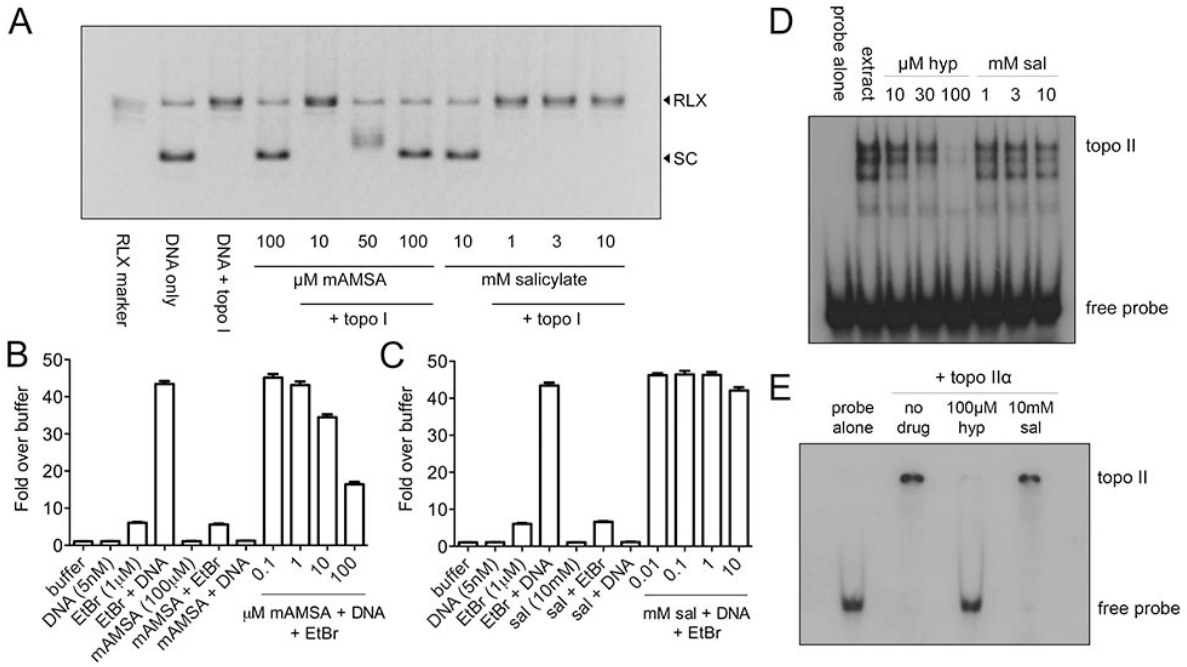
A
RLX
SC
RLX marker
DNA only
DNA + topo I
100 10 50 100 10 1 3 10
μM mAMSA
mM salicylate
+ topo I
+ topo I
B
Fold over buffer
buffer
DNA (5nM)
EtBr (1μM)
EtBr + DNA
mAMSA (100μM)
mAMSA + EtBr
mAMSA + DNA
0.1 1 10 100
μM mAMSA + DNA + EtBr
C
Fold over buffer
buffer
DNA (5nM)
EtBr (1μM)
EtBr + DNA
sal (10mM)
sal + EtBr
sal + DNA
0.01 0.1 1 10
mM sal + DNA + EtBr
D
probe alone
extract
μM hyp
mM sal
10 30 100 1 3 10
topo II
free probe
E
+ topo IIα
probe alone
no drug
100μM hyp
10mM sal
topo II
free probe

Figure 4.1. 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. A representative gel from at least three different experiments is shown. (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 [^{32}P]-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. A representative autoradiograph from at least three different experiments is shown. (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) with a representative autoradiograph from at least three different experiments shown.

concentrations of salicylate did not alter the mobility of the relaxed topoisomers, indicating that salicylate does not intercalate DNA (Fig. 4.1A).

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. 4.1B), indicative of ethidium bromide displacement. In contrast, the addition of increasing concentrations of salicylate did not alter the intensity of ethidium bromide fluorescence (Fig. 4.1C). 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. 4.1D) (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. 4.1). 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. 4.1D). In contrast, addition of increasing concentrations of salicylate had no effect on either the formation or mobility of the topo II-DNA complex (Fig. 4.1D), 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 4.1E, 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. 4.1D).

4.4.2 Topoisomerase 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 *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. 4.2A). 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. 4.2A). Addition of salicylate led to a dose-dependent reduction in topo IIα-mediated ATP hydrolysis (Fig. 4.2B).

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

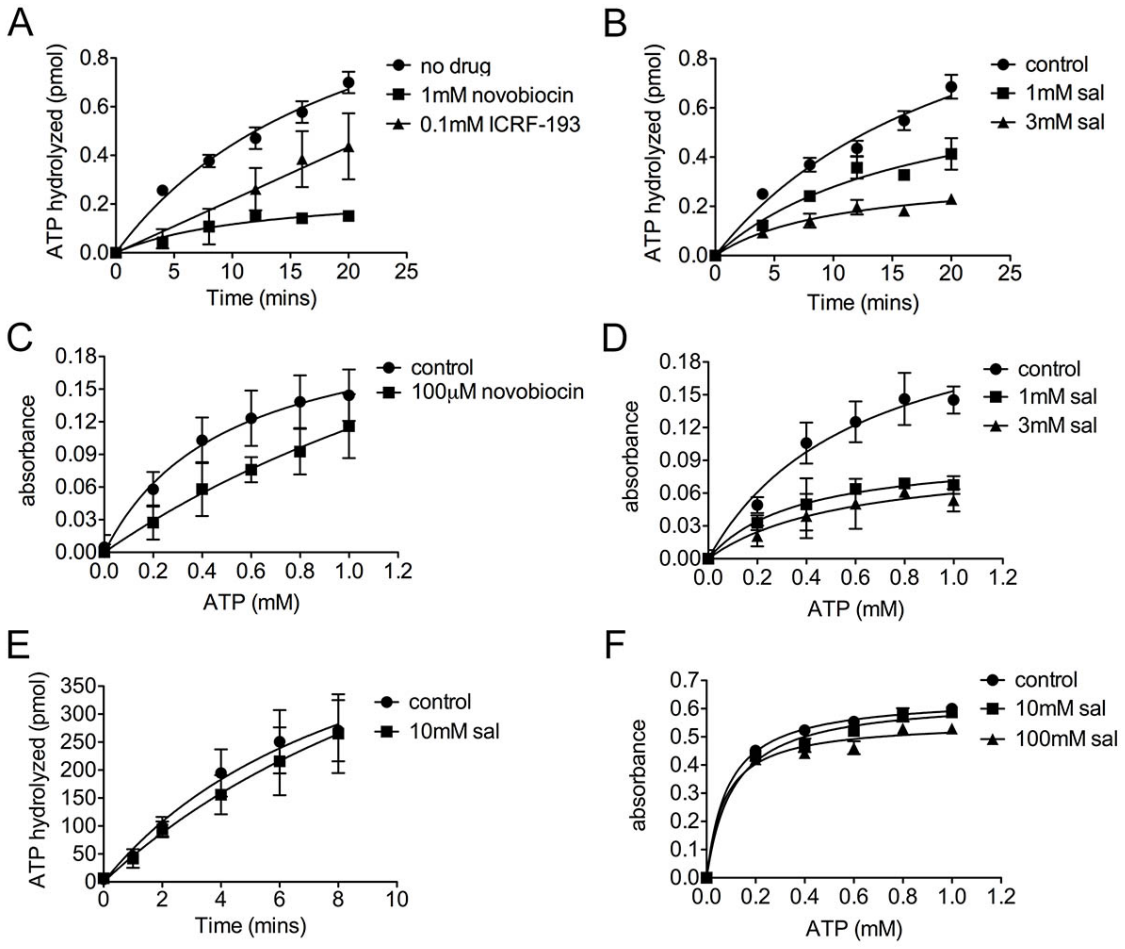
A
ATP hydrolyzed (pmol)
Time (mins)
no drug
1mM novobiocin
0.1mM ICRF-193
B
ATP hydrolyzed (pmol)
Time (mins)
control
1mM sal
3mM sal
C
absorbance
ATP (mM)
control
100μM novobiocin
D
absorbance
ATP (mM)
control
1mM sal
3mM sal
E
ATP hydrolyzed (pmol)
Time (mins)
control
10mM sal
F
absorbance
ATP (mM)
control
10mM sal
100mM sal

Figure 4.2. Salicylate non-competitively inhibits topoisomerase 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. In all experiments, data represent the mean and SEM of at least 4 independent replicates.

by novobiocin (Fig. 4.2C). 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. 4.2D). 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. 4.2E and 4.2F).

4.4.3 Salicylate does not promote formation of salt-stable topoisomerase 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. 4.3; 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

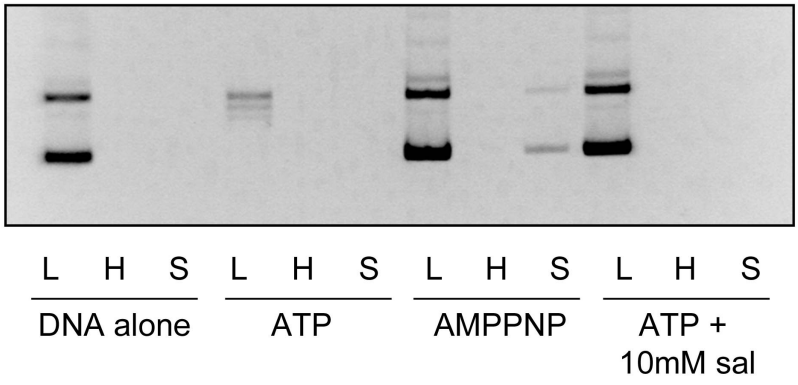

Figure 4.3. Topoisomerase IIα closed clamps are not stabilized by salicylate.

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^{o}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. Four independent experiments were performed and a representative gel is shown.

species on the DNA gel (Fig. 4.3; 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. 4.3; AMPPNP, lane S). Although addition of salicylate led to an inhibition of DNA relaxation (Fig. 4.3; ATP + 10 mM 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. 4.3; ATP + 10mM sal, lane S).

4.4.4 Salicylate inhibits the cleavage reaction of topoisomerase 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. 4.4A and 4.4B). 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. 4.4A and 4.4C). 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 et al., 1993). 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. 4.4D and 4.4E). 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-

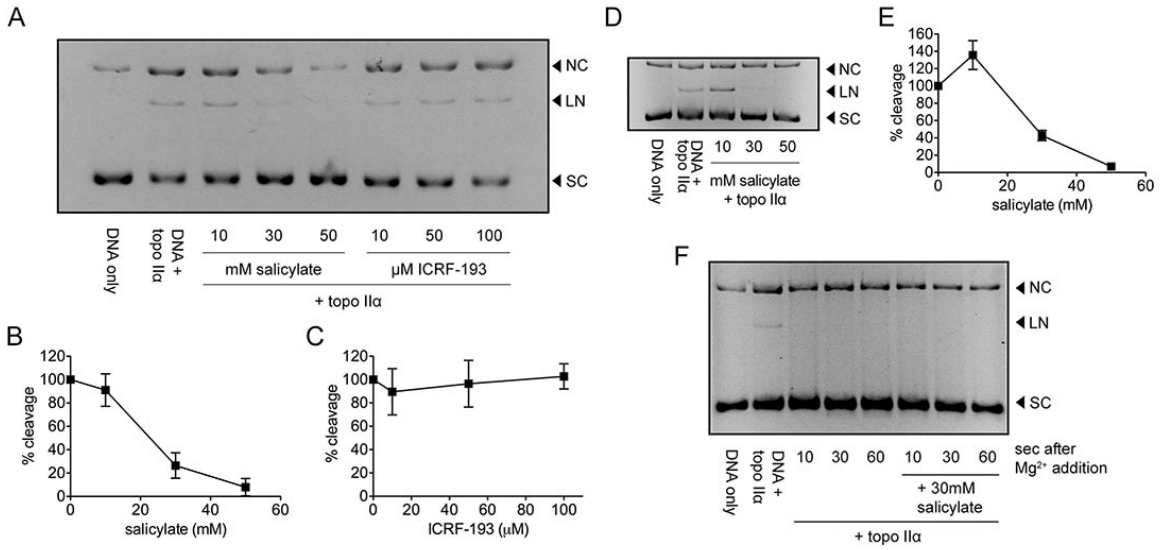
A
NC
LN
SC
DNA only
DNA + topo IIα
10 30 50 10 50 100
mM salicylate
μM ICRF-193
+ topo IIα
B
% cleavage
salicylate (mM)
C
% cleavage
ICRF-193 (μM)
D
NC
LN
SC
DNA only
DNA + topo IIα
10 30 50
mM salicylate + topo IIα
E
% cleavage
salicylate (mM)
F
NC
LN
SC
DNA only
DNA + topo IIα
10 30 60 10 30 60
sec after Mg2+ addition
+ 30mM salicylate
+ topo IIα

Figure 4.4. Salicylate inhibits topoisomerase 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 Ca^{2+}. 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). The graph represents the mean and standard deviations of three independent experiments. (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 Ca^{2+} in the absence of ATP. After addition of salicylate, reactions were reinitiated with Mg^{2+} and trapped at the indicated time points and examined as in (A). A representative image from three independent experiments is shown. SC, supercoiled DNA; LN, linear DNA; NC, nicked circular DNA.

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. 4.4F). Our experiments demonstrate that the religation of DNA occurs almost instantaneously and is not impeded in the presence of 30 mM salicylate, a concentration that severely impairs DNA cleavage.

4.4.5 Salicylate preferentially inhibits the alpha isoform of human topoisomerase 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. 4.5A). To determine if preferential inhibition was observed when assessing pre-strand passage, we repeated the plasmid cleavage assay in the presence of Ca^{2+} and the absence of ATP with purified human topo IIβ (Fig. 4.5B and 4.5C). 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.

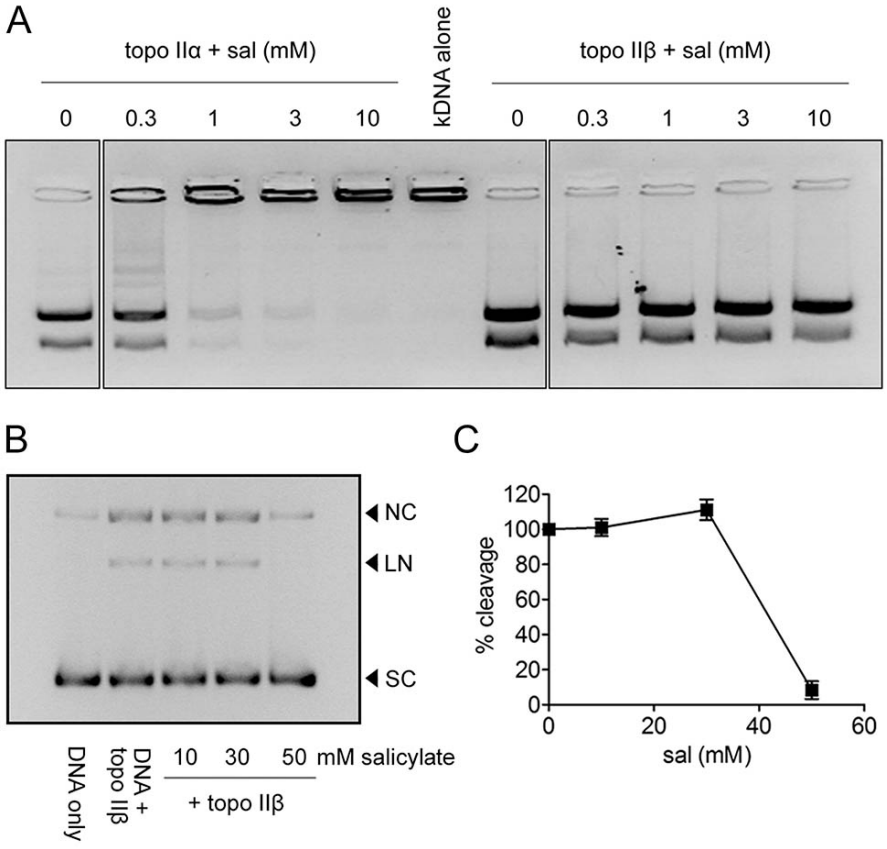
A
topo IIα + sal (mM)
0
0.3
1
3
10
kDNA alone
topo IIβ + sal (mM)
0
0.3
1
3
10
B
NC
LN
SC
DNA only
DNA + topo IIβ
10
30
50 mM salicylate
+ topo IIβ
C
% cleavage
120
100
80
60
40
20
0
0
20
40
60
sal (mM)

Figure 4.5. Salicylate selectively inhibits the topoisomerase 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. A gel representative of at least three independent experiments is shown. (B) Topo IIβ was incubated in a reaction containing plasmid DNA, 5 mM $CaCl_2$ 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.

4.5 Discussion

Compounds that disrupt the catalytic activity of topo II are broadly classed with multiple mechanisms of action (Fig. 4.6). 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. 4.1). 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. 4.1).

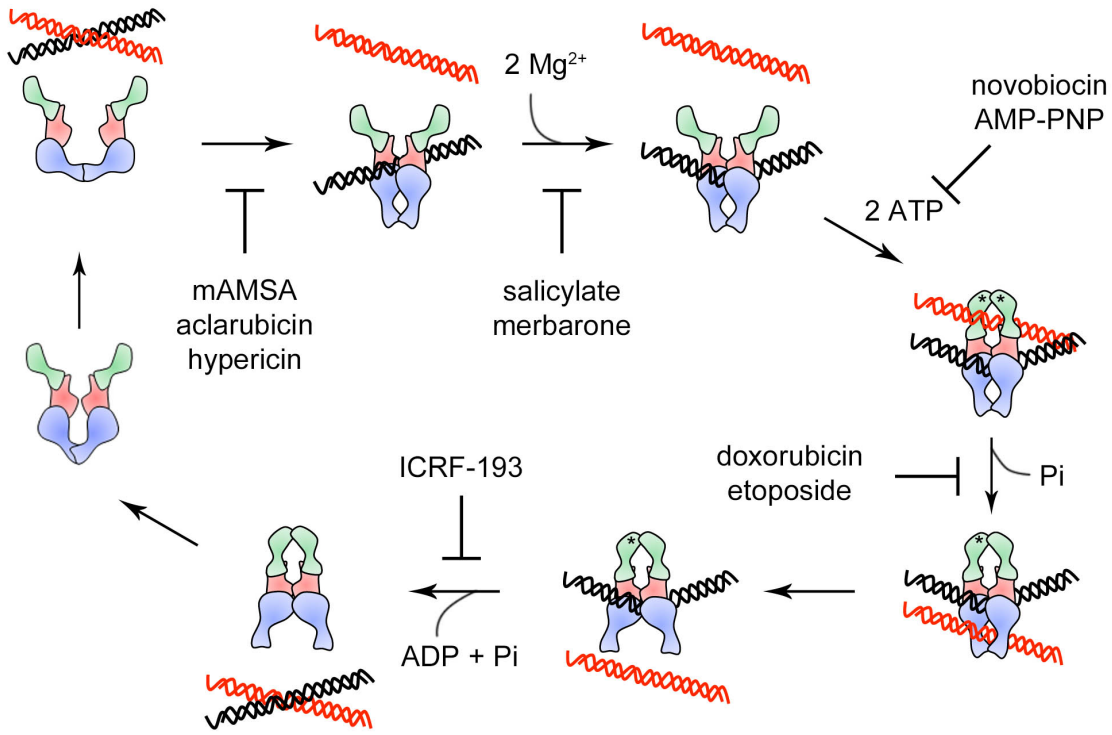
2 Mg2+
novobiocin
AMP-PNP
2 ATP
mAMSA
aclarubicin
hypericin
salicylate
merbarone
doxorubicin
etoposide
Pi
ICRF-193
ADP + Pi

Figure 4.6. Model for salicylate-mediated inhibition of topoisomerase 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 Mg^{2+}-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.

We observed that salicylate decreases topo IIα-mediated ATP hydrolysis (Fig. 4.2B). This inhibition was non-competitive, as it was not overcome by increasing the concentration of ATP (Fig. 4.2D); 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, novobiocin (Fig. 4.2C) 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. 4.3), even at high (100 mM) concentrations (unpublished observations). We did, however, observe complete inhibition of topo IIα catalytic activity (Fig. 4.3; ATP + 10 mM 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. 4.2) 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. 4.4D and 4.4E). 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 4.4F). 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. 4.5). 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., 2012). 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.

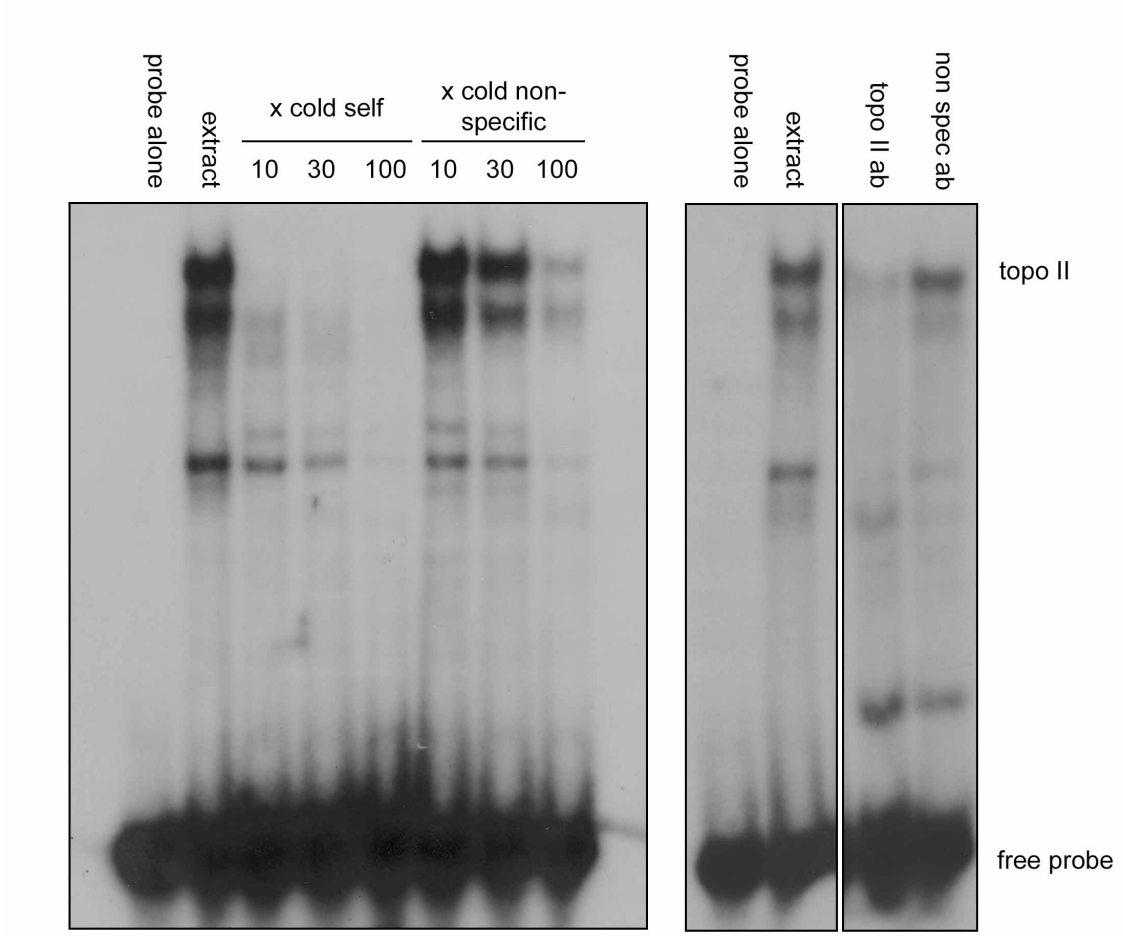
probe alone
extract
x cold self
x cold non-specific
10
30
100
10
30
100
probe alone
extract
topo II ab
non spec ab
topo II
free probe

Figure 4.S1. Supplemental figure.

Nuclear extracts were prepared from MCF-7 cells and incubated with a [α-^{32}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 IIα in the observed binding complex was confirmed by supershift assay with the addition of either a topo IIα-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. The experiment was repeated three times and a representative gel is shown for each.

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Chapter Five: Structural determinants of the catalytic inhibition of human topoisomerase IIα by salicylate analogs and salicylate-based drugs

In early experiments, including those described in Chapter 3, we observed a significant difference between the *in vitro* potencies of topo II inhibition of benzoate and salicylate. Thus, we hypothesized that an examination of structural derivatives of salicylate would yield novel insights into the chemical determinants dictating potency of topo II inhibition by salicylate-based compounds. Our studies determined that the 2'- and 5'-positions of salicylate play key roles for determining potency of topo IIα inhibition and that clinically used salicylate-based pharmaceuticals function as effective inhibitors of topo II.

The complete findings are contained in the following report[1]. The manuscript has been reproduced in its entirety, but with the sections, figures and references reformatted to adhere to the guidelines outlined by the University of Calgary Faculty of Graduate Studies.

[1] Bau JT and Kurz EU (2014) Structural determinants of the catalytic inhibition of human topoisomerase IIα by salicylate analogs and salicylate-based drugs. Biochem. Pharmacol. 89:464-476.

5.1 Abstract

We previously identified salicylate as a novel catalytic inhibitor of human DNA topoisomerase II (topo II; EC 5.99.1.3) that preferentially targets the alpha isoform by interfering with topo II-mediated DNA cleavage. Many pharmaceuticals and compounds found in foods are salicylate-based. We have now investigated whether these are also catalytic inhibitors of topo II and the structural determinants modulating these effects. We have determined that a number of hydroxylated benzoic acids attenuate doxorubicin-induced DNA damage signaling mediated by the ATM protein kinase and inhibit topo II decatenation activity *in vitro* with varying potencies. Based on the chemical structures of these and other derivatives, we identified unique properties influencing topo II inhibition, including the importance of substitutions at the 2'- and 5'-positions. We extended our findings to a number of salicylate-based pharmaceuticals including sulfasalazine and diflunisal and found that both were effective at attenuating doxorubicin-induced DNA damage signaling, topo II DNA decatenation and they blocked stabilization of doxorubicin-induced topo II cleavable complexes in cells. In a manner similar to salicylate, we determined that these agents inhibit topo II-mediated DNA cleavage. This was accompanied by a concomitant decrease in topo II-mediated ATP-hydrolysis. Taken together, these findings reveal a novel function for the broader class of salicylate-related compounds and highlight the need for additional studies into whether they may impact the efficacy of chemotherapy regimens that include topo II poisons.

5.2 Introduction

Topoisomerase II (topo II; EC 5.99.1.3) is a ubiquitous and essential enzyme required for the regulation of DNA topology in cells (Nitiss, 2009a; Schoeffler and Berger,

2008). The regulation of the topological structure of DNA is necessary for replication and transcription, and for proper segregation of chromosomes during mitosis. While lower eukaryotes express one topo II, mammalian cells express two isoforms (topo IIα and topo IIβ), with each responsible for distinct roles within the cell (Nitiss, 2009a; Schoeffler and Berger, 2008). Topo IIα has key roles in cell division and DNA replication, while topo IIβ is important in regulated transcription and neuronal development (Ju et al., 2006; Yang et al., 2000).

Targeting of topo II has proven to be an effective approach in cancer chemotherapy (Nitiss, 2009b; Pommier, 2013). Agents targeting topo II include the widely used drugs doxorubicin and etoposide, termed topo II 'poisons' for their ability to stabilize the topo II-DNA cleavable complex resulting in the accumulation of DNA double-stranded breaks. The accumulation of these breaks eventually overwhelms the cell's DNA repair capacity leading to cell death. In contrast, catalytic inhibitors impair topo II activity without stabilizing the cleavable complex and act at any one of several steps within the topo II catalytic cycle (Andoh and Ishida, 1998; Larsen et al., 2003). This includes blocking the binding of topo II to DNA, inhibiting DNA cleavage, and preventing the dissociation of the enzyme from DNA following strand passage. As a result, catalytic inhibitors do not generate topo II-mediated DNA double-stranded breaks (Larsen et al., 2003). While topo II poisons have been long-time mainstays of many chemotherapy regimens, catalytic inhibitors have achieved more limited clinical utility, primarily in reducing the cardiotoxicity associated with doxorubicin administration (Lipshultz et al., 2010, 2013; Vrooman et al., 2011).

We previously identified salicylate (chemically defined as 2-hydroxybenzoate (2-HBA)) as a novel catalytic inhibitor of topo IIα and determined that it selectively inhibits

the α isoform (Bau and Kurz, 2011; Bau et al., 2014). Salicylate blocks topo II ATPase activity through a non-competitive mechanism, which we have determined occurs secondary to interfering with DNA cleavage (Bau et al., 2014). Furthermore, we have shown that the effects of salicylate are independent of its actions as an antioxidant, as an inhibitor of NFκB or cyclooxygenases, and are specific for topo II (Bau and Kurz, 2011). Critically, pretreatment of cells with salicylate prevents doxorubicin-induced DNA double-stranded breaks and reduces the efficacy of both doxorubicin and etoposide in cyto (Bau and Kurz, 2011).

Salicylates are commonly found in foods derived from plants, cosmetics and both over-the-counter and prescription pharmaceuticals, including aspirin (acetylsalicylic acid; ASA), methyl salicylate (oil of wintergreen), sulfasalazine and diflunisal. Salicylate is the major metabolite of aspirin, which forms spontaneously after rapid hydrolysis of the parent compound or after transfer of the acetyl group to a cyclooxygenase (Lecomte et al., 1994). Now common for its daily use in the prevention of cardiovascular and cerebrovascular events, in addition to being used for its anti-inflammatory, anti-pyretic and analgesic effects, it is estimated that almost 40,000 metric tonnes of aspirin are ingested annually worldwide (Warner and Mitchell, 2002). Many compounds found in edible plants and spices bear structural similarity to salicylate (Duthie and Wood, 2011; Paterson et al., 2006) and diets rich in these foods can significantly increase plasma levels of salicylate (Paterson et al., 2006). Thus, our prior observations with salicylate raise the possibility that these compounds may also be catalytic inhibitors of topo II. Furthermore, in our previous work we observed that salicylate more potently attenuates doxorubicin-induced DNA damage signaling when compared with benzoate, despite differing by only a single hydroxyl moiety

(Bau and Kurz, 2011). This illustrates that small ring modifications can have significant biochemical effects with regards to topo II inhibition.

In this study, we describe a systematic evaluation of benzoic acid derivatives bearing modifications varying in number, position, size and/or electronegativity and identify the structural modifications important for topo IIα inhibition. Furthermore, we have extended our observations to identify a broader class of topo II catalytic inhibitors characterized by their salicylate-related structures, including the salicylate-based drugs sulfasalazine and diflunisal. Our systematic evaluation of these compounds has identified the structural requirements critical for inhibition of topo II, which, taken together, highlight the need for further studies in examining whether these compounds have deleterious consequences for those receiving topo II poison-based chemotherapy regimens.

5.3 Materials and Methods

5.3.1 Reagents

All of the chemicals used in this study (doxorubicin, salicylate (2-HBA), 3-HBA, 4-HBA, 2,3-dihydroxybenzoate (DHBA), 2,4-DHBA, 2,5-DHBA, 2,6-DHBA, 3,4-DHBA, 3,5-DHBA, 2,3,4-trihydroxybenzoate (THBA), 3,4,5-THBA, salicylamide, salicylaldehyde, 2-methylbenzoate (2-MBA), 2-fluorobenzoate (2-FBA), acetylsalicylic acid (ASA, aspirin), diflunisal, sulfasalazine, sulfapyridine, and 5-aminosalicylic acid (5-ASA)) were purchased from Sigma-Aldrich (Oakville, ON, Canada). Stock solutions of these compounds were prepared in 20% v/v DMSO (Sigma-Aldrich), with the exception of diflunisal and sulfasalazine, which were prepared in 70% v/v DMSO. In all experiments, the final concentration of DMSO was 100-fold less than the stock concentration and control samples

contained an equivalent amount of vehicle. Stock solutions of compounds purchased in the acid form were neutralized to pH 7 with NaOH prior to use in experiments. Stocks were stored in aliquots at -20°C and protected from light. Recombinant purified human topo IIα was purchased from TopoGEN (Port Orange, FL, USA). All other common laboratory chemicals were of the highest grade available and were purchased from Sigma-Aldrich, unless otherwise stated.

5.3.2 Cell culture, preparation of cell extracts and immunoblotting

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% CO_2. Whole cell extracts were prepared from logarithmically growing cells as previously described (Kurz et al., 2004). Electrophoresis and immunoblotting conditions were also as previously described (Bau and Kurz, 2011).

5.3.3 Image analysis

Densitometric analysis of immunoblots was preformed using ImageQuant (GE Healthcare Life sciences, Baie d'Urfe, QC, Canada). Intensity of phosphorylation levels was normalized to the intensity of the total pool of protein. Data were collected from at least three independent replicates and expressed as relative intensity compared with doxorubicin alone as previously described (Bau and Kurz, 2011).

5.3.4 kinetoplast DNA (kDNA) decatenation assay

The effects of the salicylate-related analogs on topo IIα catalytic activity were examined as previously described (Bau and Kurz, 2011), with the following modifications. Human topo IIα (2 U) was incubated with 200 ng kinetoplast DNA (kDNA, Topogen) in reaction buffer A (10 mM Tris pH 7.7, 50 mM NaCl, 50 mM KCl, 0.1 mM EDTA, 10 mM $MgCl_2$, 0.03 μg μL^{-1} BSA (New England Biolabs, Whitby, ON)) for 10 min on ice prior the addition of ATP (0.5 mM, disodium salt (Topogen)) 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 (Invitrogen, Burlington, ON).

5.3.5 In vivo complex of enzyme assay

The *in vivo* complex of enzyme assay was completed as described previously (Bau and Kurz, 2011; Subramanian et al., 2001).

5.3.6 ATP hydrolysis assay using [γ-^{32}P]-ATP

Topo II-mediated ATP hydrolysis using [γ-^{32}P]-ATP (PerkinElmer, Waltham, MA, USA) was assessed by thin layer chromatography as described (Bau et al., 2014).

5.3.7 Topoisomerase II-mediated DNA cleavage assay

The effects of benzoate and salicylate on topo II-mediated DNA cleavage in the presence of ATP was assessed as previously described (Bau and Kurz, 2011). ATP was included in this assay to simultaneously evaluate both pre-strand passage and post-strand passage DNA cleavage.

5.3.8 ATP-independent DNA cleavage assay

Topo II-mediated, ATP-independent DNA cleavage was examined as previously described (Bau et al., 2014).

5.4 Results

5.4.1 Addition of a hydroxyl group to benzoate increases potency of enzyme inhibition

Previously, we observed a difference between salicylate and benzoate in their relative ability to attenuate doxorubicin-induced DNA damage signaling in human MCF-7 breast cancer cells (Bau and Kurz, 2011). Despite differing from salicylate by the absence of only a single hydroxyl (-OH) group at the 2'-position, benzoate was noted to be ten-fold less effective than salicylate at attenuating doxorubicin-induced DNA damage signaling (Bau and Kurz, 2011). To examine if this difference was also observed using purified topo IIα, we tested both compounds using a cleavage assay in the presence of ATP. While topo IIα completely converts the supercoiled DNA substrate to a relaxed form in the absence of drug, relaxation was inhibited in a dose-dependent manner in the presence of salicylate, with partial inhibition observed at 3 mM salicylate and complete inhibition observed at 10 mM (Fig. 5.1A). Furthermore, no linear reaction product was formed, reaffirming our previous findings that salicylate does not stabilize the topo II-DNA cleavable complex and thus is not a topo II poison (Bau and Kurz, 2011; Bau et al., 2014). By comparison, use of benzoate in place of salicylate in the reaction also led to the

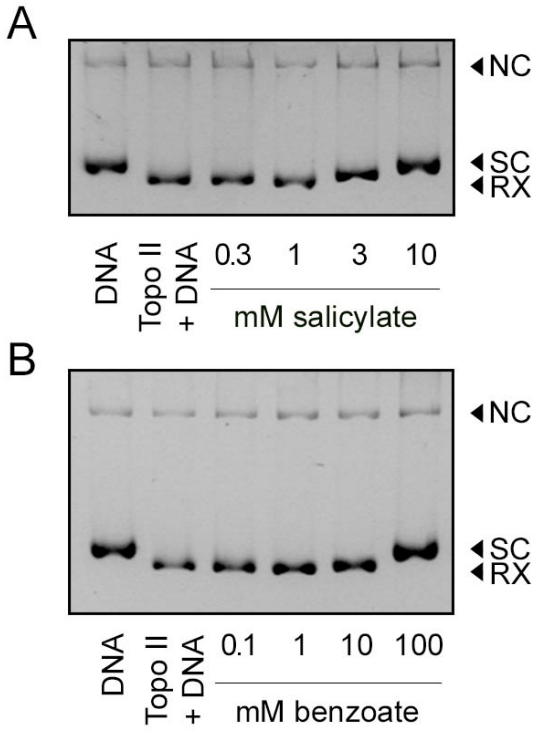


Figure 5.1. Salicylate is a more potent inhibitor of topoisomerase II catalytic activity than benzoate.

The ability of purified topo II (8 U) to relax supercoiled pBR322 plasmid DNA was examined *in vitro* in the presence of salicylate (A) or benzoate (B). Reactions were incubated for 10 min on ice prior to the addition of ATP (0.5 mM). Reactions were immediately transferred to a 37°C water bath and incubated for an additional 8 min. Reactions were stopped by the addition of SDS, EDTA and NaCl. Topo II was digested with proteinase K and reaction products were resolved overnight by agarose gel electrophoresis. NC, nicked circular DNA; SC, supercoiled DNA; RX, relaxed DNA. Each of the above experiments was conducted at least five independent times and a representative image is shown for each.

inhibition of topo II catalytic activity, but this occurred only at a concentration ten to thirty-fold higher than that required for inhibition by salicylate (Fig. 5.1B).

5.4.2 Monohydroxybenzoates comparably attenuate doxorubicin-induced DNA damage signaling

Given the observed differences in potency of topo IIα inhibition between benzoate and salicylate, we hypothesized that structurally related compounds may also be inhibitors of topo IIα and selected compounds to study based on several criteria. We sub-classified these compounds based on their chemical modifications, namely: ring modifications (Fig. 5.2A to D), modifications of the carboxylic acid moiety (Fig. 5.2E) and salicylate-based pharmaceuticals or their metabolites (Fig. 5.2F). Many of the compounds shown in Figure 2 occur naturally in plants (Paterson et al., 2006) or are clinically used (Burke et al., 2009).

We first assessed whether these compounds attenuate doxorubicin-induced DNA damage signaling in MCF-7 cells, an effective screening method we have previously established (Bau and Kurz, 2011). It is known that catalytic inhibitors can attenuate the stabilization of topo II-mediated cleavable complexes induced by topo II poisons. Poisoning of topo II by drugs such as doxorubicin results in the formation of covalent topo II-DNA adducts, which, when removed, expose DNA double-stranded breaks. These trigger a DNA double-stranded break response mediated by the ATM protein kinase and its downstream effectors including Chk2, SMC1 and p53 (Kurz et al., 2004). Compounds that attenuate the DNA damaging signaling induced by doxorubicin are characterized by a reduction in the relative phosphorylation of these effector proteins. Densitometric analysis of replicate gels allows for determination of the effectiveness of these agents at attenuating the damaging effects of doxorubicin.

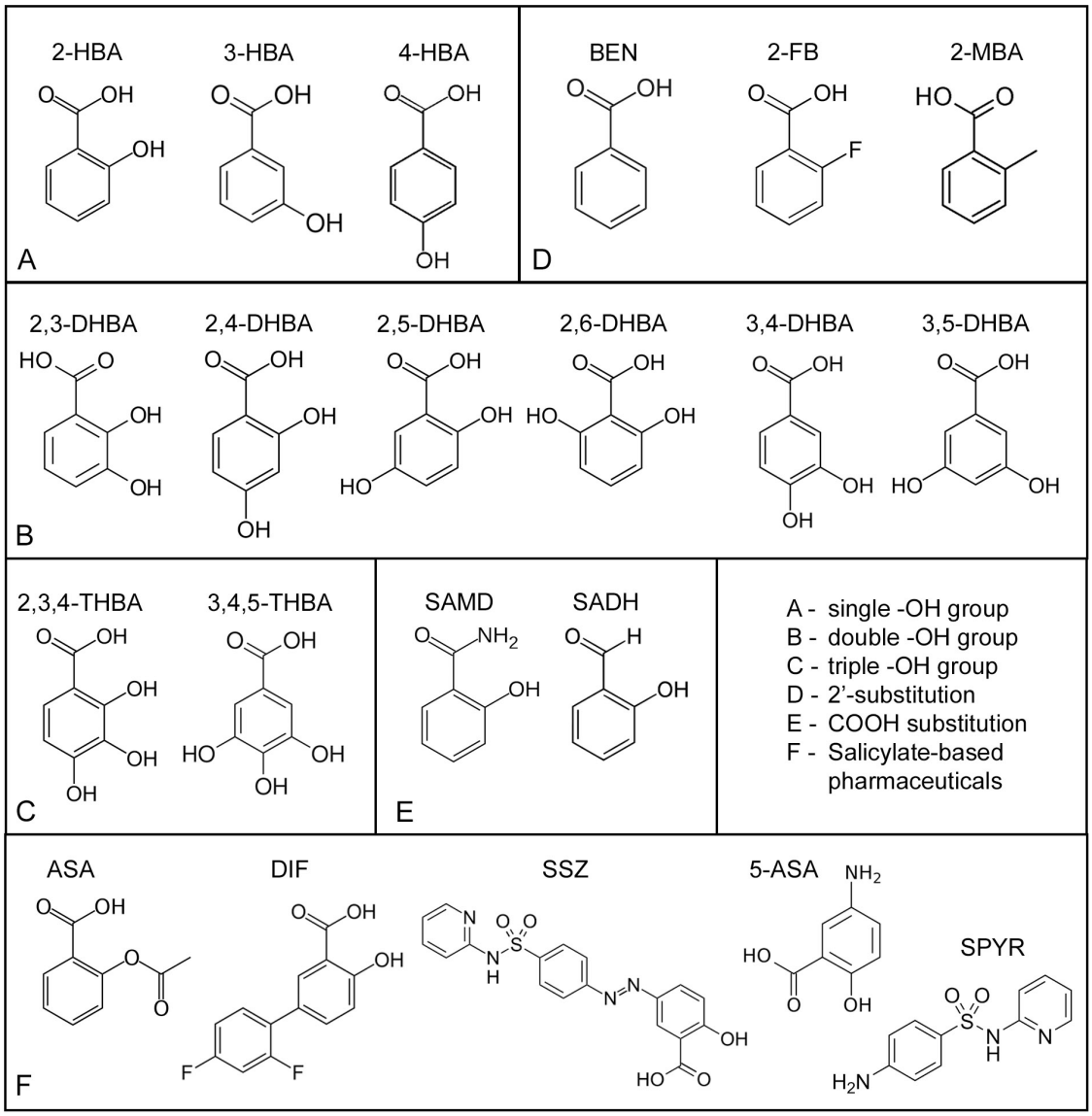

2-HBA
3-HBA
4-HBA
A
BEN
2-FB
2-MBA
D
2,3-DHBA
2,4-DHBA
2,5-DHBA
2,6-DHBA
3,4-DHBA
3,5-DHBA
B
2,3,4-THBA
3,4,5-THBA
C
SAMD
SADH
E
A - single -OH group
B - double -OH group
C - triple -OH group
D - 2'-substitution
E - COOH substitution
F - Salicylate-based pharmaceuticals
ASA
DIF
SSZ
5-ASA
SPYR
F

Figure 5.2. Salicylate derivatives examined in this study.

Derivatives were selected based on the number and placement of hydroxyl groups on the benzoic acid ring. These hydroxylated benzoic acids (HBA) were classified into single (A; HBA), double (B, DHBA) and triple (C, THBA) hydroxyl-substituted compounds. (D) Derivatives with modifications at the 2'-position of benzoate (BEN), including 2-fluorobenzoic acid (2-FBA) and 2-methyl benzoic acid (2-MBA). (E) Compounds with changes to the carboxylic acid group: salicylamide (SAMD) and salicylaldehyde (SADH). (F) Clinically-used pharmaceuticals containing a salicylate group: acetylsalicylic acid (ASA), diflunisal (DIF), and sulfasalazine (SSZ), and the metabolites of sulfasalazine: 5-aminosalicylic acid (5-ASA) and sulfapyridine (SPYR).

Given the observed difference between benzoate and salicylate (Fig 5.1 and Bau and Kurz, 2011), we hypothesized that the relative position of hydroxyl moieties around the benzoate ring could impact the potency of inhibition. Placement of a single hydroxyl group in the 3'- or 4'-position did not alter the relative attenuation of doxorubicin-induced DNA damage signaling when compared with salicylate (Fig. 5.3). This suggests that the presence of a single hydroxyl group, regardless of its position on the benzoate ring, is sufficient to enhance attenuation of doxorubicin-induced DNA damage signaling when compared with benzoate.

5.4.3 Placement and number of hydroxyl groups dictates potency of topoisomerase II inhibition

We subsequently asked if the addition of a second or third hydroxyl group could enhance the potency of the effect on doxorubicin-induced DNA damage signal attenuation. However, regardless of position, the presence of two hydroxyl moieties did not markedly alter the attenuation of doxorubicin-induced, ATM-mediated DNA damage signaling when compared with salicylate (Fig. 5.4). In contrast, addition of a third hydroxyl group (2,3,4-THBA) significantly enhanced the effect; pretreatment of cells with 2,3,4-THBA led to attenuation of doxorubicin-induced DNA damage signaling at a concentration ten-fold lower than that required for a comparable effect with salicylate (Figs. 5.5A and B). However, this increased potency was not observed with 3,4,5-THBA (Figs. 5.5C and D). Furthermore, at higher concentrations (above 1 mM), 3,4,5-THBA alone induced phosphorylation of the ATM, SMC1, p53 and Chk2 (data not shown). This difference between 2,3,4-THBA and 3,4,5-THBA suggests that increasing the number of hydroxyl

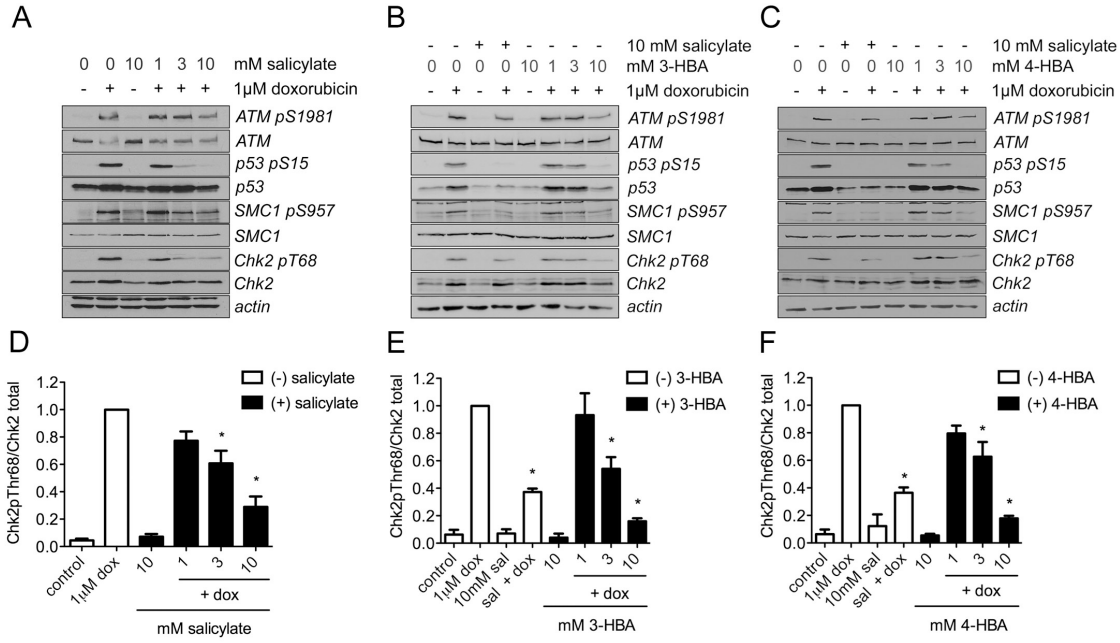

A
0 0 10 1 3 10 mM salicylate
- + - + + + 1μM doxorubicin
ATM pS1981
ATM
p53 pS15
p53
SMC1 pS957
SMC1
Chk2 pT68
Chk2
actin
B
- - + + - - - - 10 mM salicylate
0 0 0 0 10 1 3 10 mM 3-HBA
- + - + - + + + 1μM doxorubicin
ATM pS1981
ATM
p53 pS15
p53
SMC1 pS957
SMC1
Chk2 pT68
Chk2
actin
C
- - + + - - - - 10 mM salicylate
0 0 0 0 10 1 3 10 mM 4-HBA
- + - + - + + + 1μM doxorubicin
ATM pS1981
ATM
p53 pS15
p53
SMC1 pS957
SMC1
Chk2 pT68
Chk2
actin
D
Chk2pThr68/Chk2 total
(-) salicylate
(+) salicylate
control
1μM dox
+ dox
mM salicylate
E
Chk2pThr68/Chk2 total
(-) 3-HBA
(+) 3-HBA
control
1μM dox
10mM sal
sal + dox
+ dox
mM 3-HBA
F
Chk2pThr68/Chk2 total
(-) 4-HBA
(+) 4-HBA
control
1μM dox
10mM sal
sal + dox
+ dox
mM 4-HBA

Figure 5.3. Attenuation of doxorubicin-induced DNA damage signaling is observed with multiple monohydroxylated benzoates.

Human breast cancer cells (MCF-7) were treated with increasing concentrations of 2-hydroxybenzoate (salicylate (sal); A), 3-hydroxybenzoate (3-HBA; B) or 4-HBA (C) for 1 h prior to the addition of doxorubicin (dox; 1 μM) and continued incubation for an additional 2 h. Whole cell extracts were prepared and immunoblotted with phosphospecific and pan-specific antisera to ATM (phosphorylated on serine 1981), p53 (phosphorylated on serine 15), SMC1 (phosphorylated on serine 957) and Chk2 (phosphorylated on threonine 68). Immunoblotting for actin was used as a loading control. Representative immunoblots are shown for each compound. The blots of phosphorylated and total pools of Chk2 from three independent replicates for each compound were scanned, analysed densitometrically and plotted (salicylate (D), n=3; 3-HBA (E), n=3; 4-HBA (F), n=3). The error bars represent the S.E.M. Statistical significance of pretreatment effects compared with doxorubicin alone was determined by one-way ANOVA with a Tukey post-hoc test and is denoted by the asterisk (*; $p < 0.05$).

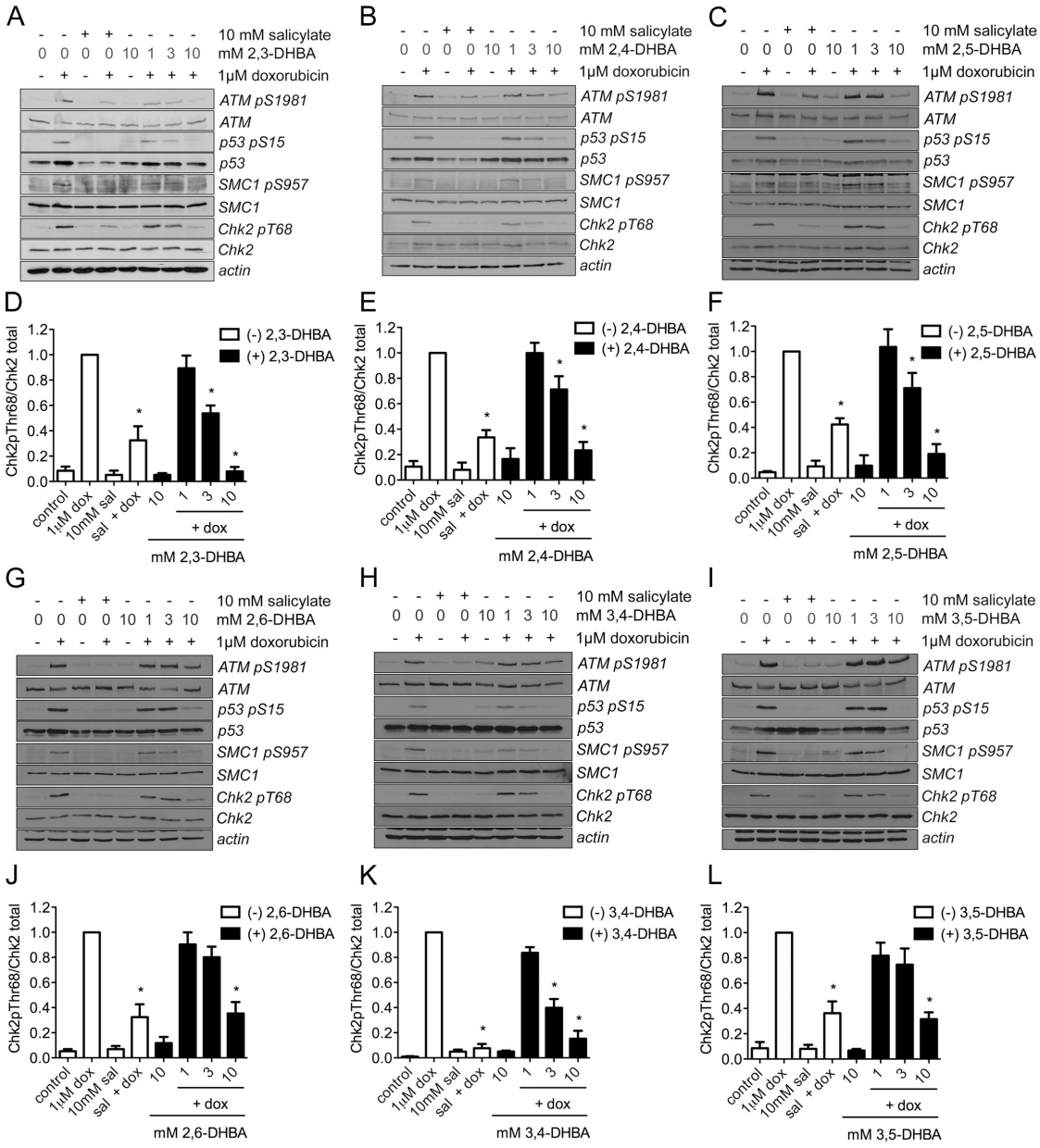
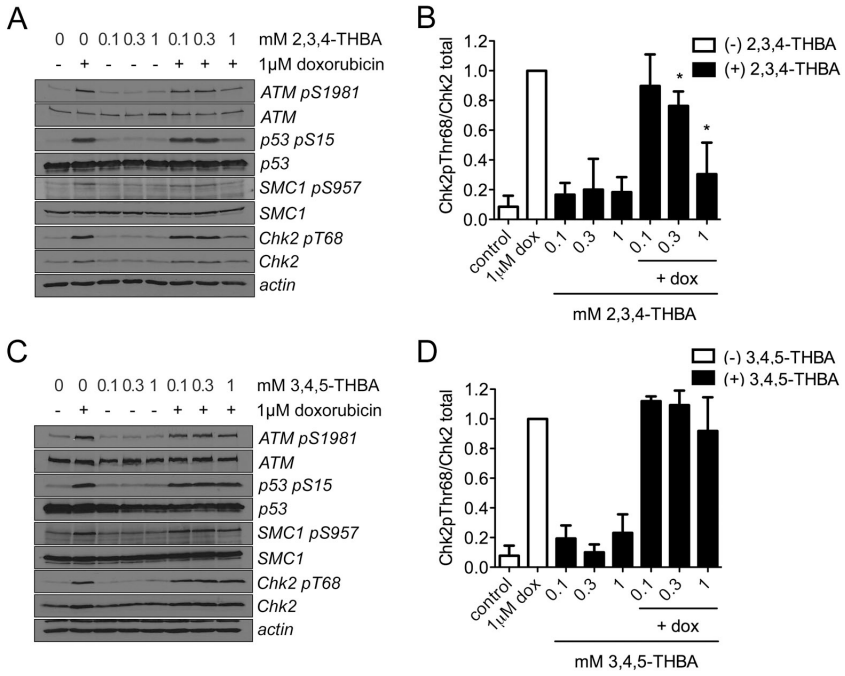

A
10 mM salicylate
mM 2,3-DHBA
1μM doxorubicin
ATM pS1981
ATM
p53 pS15
p53
SMC1 pS957
SMC1
Chk2 pT68
Chk2
actin
B
10 mM salicylate
mM 2,4-DHBA
1μM doxorubicin
C
10 mM salicylate
mM 2,5-DHBA
1μM doxorubicin
D
Chk2pThr68/Chk2 total
(-) 2,3-DHBA
(+) 2,3-DHBA
control
1μM dox
10mM sal
sal + dox
+ dox
mM 2,3-DHBA
E
(-) 2,4-DHBA
(+) 2,4-DHBA
mM 2,4-DHBA
F
(-) 2,5-DHBA
(+) 2,5-DHBA
mM 2,5-DHBA
G
10 mM salicylate
mM 2,6-DHBA
1μM doxorubicin
H
10 mM salicylate
mM 3,4-DHBA
1μM doxorubicin
I
10 mM salicylate
mM 3,5-DHBA
1μM doxorubicin
J
(-) 2,6-DHBA
(+) 2,6-DHBA
mM 2,6-DHBA
K
(-) 3,4-DHBA
(+) 3,4-DHBA
mM 3,4-DHBA
L
(-) 3,5-DHBA
(+) 3,5-DHBA
mM 3,5-DHBA

Figure 5.4. Attenuation of doxorubicin-induced DNA damage signaling is observed with dihydroxylated benzoates.

Human breast cancer cells (MCF-7) were treated with salicylate (sal; 10 mM) or increasing concentrations of dihydroxybenzoates (2,3-DHBA, n=5 (A); 2,4-DHBA, n=5 (B); 2,5-DHBA, n=4 (C); 2,6-DHBA, n=6 (G); 3,4-DHBA, n=4 (H); 3,5-DHBA, n=5 (I)) for 1 h prior to the addition of doxorubicin (dox; 1 μM) and continued incubation for an additional 2 h. Whole cell extracts were prepared, immunoblotted and analyzed as described in Fig. 5.3. (Panels D-F and J-L). Representative immunoblots are shown for each compound. The error bars represent the S.E.M. Statistical significance of pretreatment effects compared with doxorubicin alone was determined by one-way ANOVA with a Tukey post-hoc test and is denoted by the asterisk (*; $p < 0.05$).


A
0 0 0.1 0.3 1 0.1 0.3 1 mM 2,3,4-THBA
- + - - - + + + 1μM doxorubicin
ATM pS1981
ATM
p53 pS15
p53
SMC1 pS957
SMC1
Chk2 pT68
Chk2
actin
B
Chk2pThr68/Chk2 total
(-) 2,3,4-THBA
(+) 2,3,4-THBA
control
1μM dox
0.1
0.3
1
0.1
0.3
1
+ dox
mM 2,3,4-THBA
C
0 0 0.1 0.3 1 0.1 0.3 1 mM 3,4,5-THBA
- + - - - + + + 1μM doxorubicin
ATM pS1981
ATM
p53 pS15
p53
SMC1 pS957
SMC1
Chk2 pT68
Chk2
actin
D
Chk2pThr68/Chk2 total
(-) 3,4,5-THBA
(+) 3,4,5-THBA
control
1μM dox
0.1
0.3
1
0.1
0.3
1
+ dox
mM 3,4,5-THBA

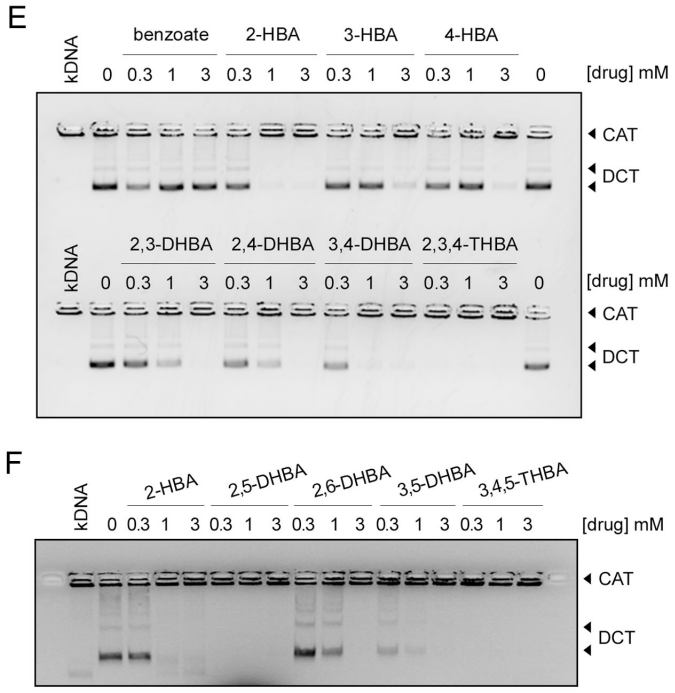

E
kDNA
benzoate
2-HBA
3-HBA
4-HBA
0 0.3 1 3 0.3 1 3 0.3 1 3 0.3 1 3 0
[drug] mM
CAT
DCT
2,3-DHBA
2,4-DHBA
3,4-DHBA
2,3,4-THBA
F
kDNA
2-HBA
2,5-DHBA
2,6-DHBA
3,5-DHBA
3,4,5-THBA
0 0.3 1 3 0.3 1 3 0.3 1 3 0.3 1 3 0.3 1 3
[drug] mM
CAT
DCT

Figure 5.5. Hydroxylated benzoates strongly inhibit topoisomerase II catalytic activity *in vitro*.

The trihydroxybenzoates (THBA), 2,3,4-THBA (n=3; A, B) and 3,4,5-THBA (n=3; C, D), were examined and analyzed as in Fig. 5.3. For each compound, representative immunoblots are shown. The error bars represent the S.E.M. Statistical significance of pretreatment effects compared with doxorubicin alone was determined by one-way ANOVA with a Tukey post-hoc test and is denoted by the asterisk (*; $p < 0.05$). (E, F) Hydroxylated benzoates were tested for their ability to inhibit topo II decatenation activity *in vitro*. Catenated kDNA (CAT) becomes decatenated (DCT) when incubated with catalytically active topo II. Reaction products were resolved by agarose gel electrophoresis and stained with ethidium bromide. Representative gels of at least three independent experimental replicates are shown.

groups alone does not enhance potency, but rather placement is important in determining the relative effect.

To examine whether these compounds inhibit the catalytic activity of topo IIα, we performed *in vitro* kDNA decatenation assays using purified recombinant human topo IIα (Fig. 5.5E and F). At the concentrations tested, benzoate was unable to inhibit topo II catalytic activity. While inhibition of decatenation was observed at 3 mM with all hydroxylated compounds tested, in the presence of lower drug concentrations differences in potency were revealed. Using this cell-free assay, salicylate (2-HBA) was observed to be at least three-fold more potent than either 3-HBA or 4-HBA. In replicate experiments, salicylate was also observed to be more potent that 2,3-DHBA, 2,4-DHBA and 2,6-DHBA, equipotent with 3,4-DHBA and less potent that either 2,5-DHBA or 3,5-DHBA at inhibiting topo II catalytic activity *in vitro*. In contrast to their differing behaviours in attenuating doxorubicin-induced DNA damage signaling (Figs. 5.5A-D), using the kDNA decatenation assay, both 2,3,4-THBA and 3,4,5-THBA were observed to be potent inhibitors of topo IIα catalytic activity, with complete inhibition observed at 0.3 mM (Figs. 5.5E and F).

5.4.4 Modification of the 2' position affects topoisomerase II inhibition

Given the effects observed with substitutions at the 2' position (2-HBA>3-HBA=4-HBA; 2,3,4-THBA >> 3,4,5-THBA), we selected several derivatives bearing alternate substitutions at this position (Fig. 5.2D). As the addition of a hydroxyl group to form salicylate leads to changes in both substituent size and electronegativity when compared with benzoate, we selected compounds that independently differed in these characteristics.

We first examined 2-FBA; fluorine is the most electronegative element and can act as a hydrogen bond acceptor, while hydroxyl groups act as donors. In MCF-7 cells, 2-FBA was weaker than salicylate at attenuating doxorubicin-induced DNA damage signaling (Figs. 5.6A and B). In a similar manner, placement of a methyl group at the 2'-position (2-MBA), a substituent that is both larger and non-polar compared with hydroxyl, was also less effective than salicylate in attenuating doxorubicin-mediated DNA damage signaling (Figs. 5.6C and D). However, while 2-FBA inhibited topo II catalytic activity *in vitro*, 2-MBA was unable to do so (Fig. 5.6G).

ASA (aspirin) also contains a modification at the 2'-position. In ASA, the ester group at the 2'-position is not only larger, but acts as a hydrogen bond acceptor at multiple positions. We observed that ASA was less potent than salicylate at low concentrations at attenuating doxorubicin-induced DNA damage signaling, although attenuation was observed at 10 mM ASA (Figs. 5.6E and F). In keeping with this reduced potency, ASA was also less effective at inhibiting topo II activity *in vitro* (Fig 5.6G).

5.4.5 Modification of the carboxylate moiety does not alter the ability to inhibit topoisomerase II

The carboxylate group of salicylate provides opportunities for interactions with neighboring moieties. Thus, we compared the ability of salicylamide and salicylaldehyde to decrease doxorubicin-induced DNA damage signaling in MCF-7 cells. Salicylamide, a salicylate-based pharmaceutical, effectively attenuated doxorubicin-induced DNA damage signaling and inhibited enzyme activity *in vitro* (Fig. 5.7). Salicylaldehyde was too toxic for use on MCF-7 cells to evaluate its effects on doxorubicin-induced DNA damage

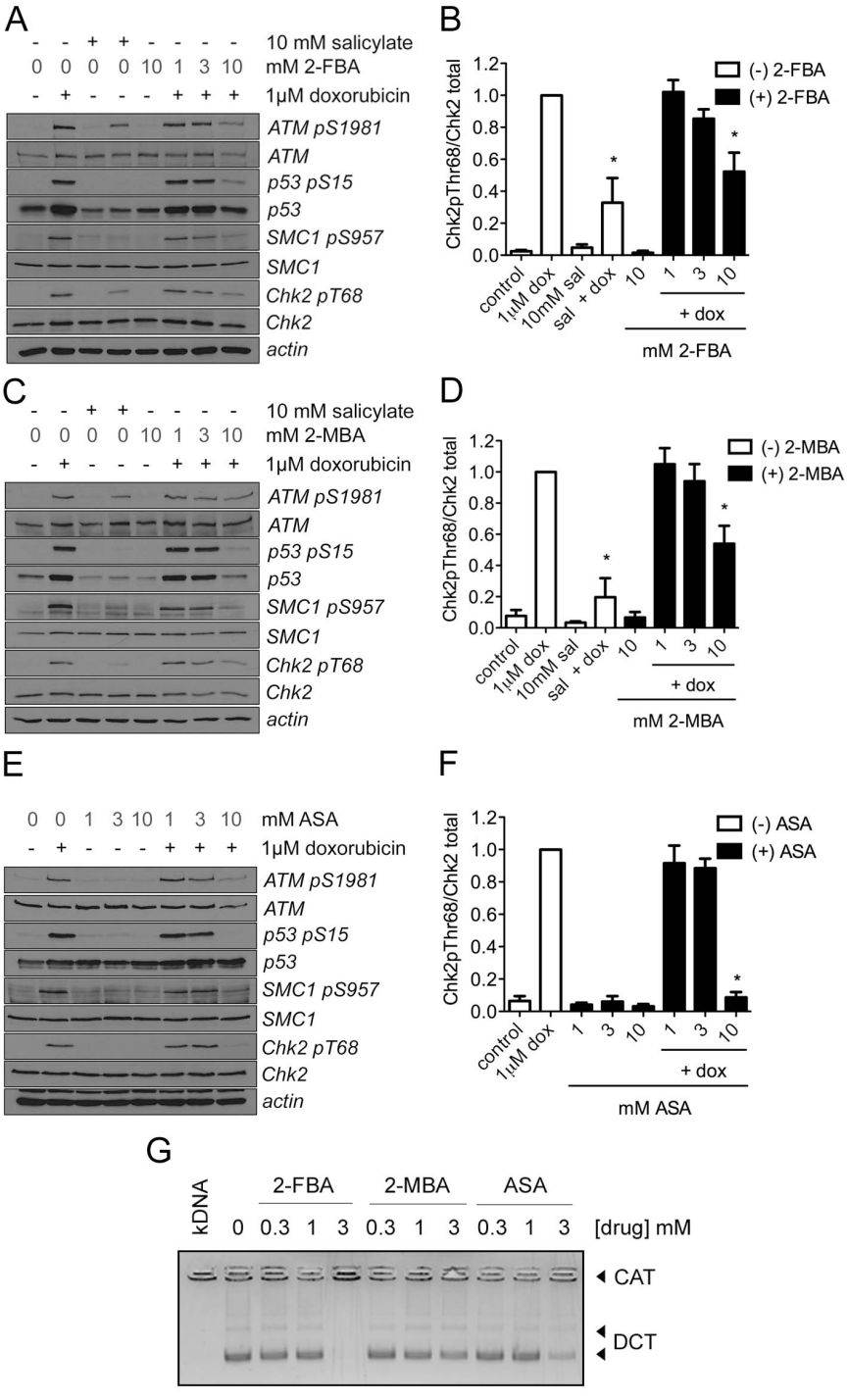

A
10 mM salicylate
mM 2-FBA
1μM doxorubicin
ATM pS1981
ATM
p53 pS15
p53
SMC1 pS957
SMC1
Chk2 pT68
Chk2
actin
B
Chk2pThr68/Chk2 total
(-) 2-FBA
(+) 2-FBA
control
1μM dox
10mM sal
sal + dox
+ dox
mM 2-FBA
C
10 mM salicylate
mM 2-MBA
1μM doxorubicin
D
(-) 2-MBA
(+) 2-MBA
mM 2-MBA
E
mM ASA
1μM doxorubicin
F
(-) ASA
(+) ASA
mM ASA
G
kDNA
2-FBA
2-MBA
ASA
[drug] mM
CAT
DCT

Figure 5.6. Substitution at the 2'-position of benzoate decreases the effectiveness of topoisomerase II catalytic inhibition.

Benzoate derivatives bearing modifications at the 2'-position were examined for their ability to attenuate doxorubicin-induced DNA damage signaling. MCF-7 human breast cancer cells were pretreated with salicylate (sal; 10 mM) or increasing concentrations of 2-fluorobenzoate (2-FBA, n=3; A, B), 2-methylbenzoate (2-MBA, n=3; C, D), or acetylsalicylic acid (ASA, n=4; E, F) for 1 h prior to the addition of doxorubicin (dox; 1 μM) and continued incubation for an additional 2 h. For all treatments, whole cell extracts were prepared, immunoblotted and analyzed as in Fig. 5.3. Representative immunoblots are shown for each compound. The error bars represent the S.E.M. Statistical significance of pretreatment effects compared with doxorubicin alone was determined by one-way ANOVA with a Tukey post hoc test (*; $p < 0.01$). (G) Purified topo II was incubated with catenated kDNA in the presence of the indicated compounds. Reaction products were resolved by agarose gel electrophoresis and stained with ethidium bromide. A gel representative of three independent experimental replicates is shown. Catenated kDNA, CAT; decatenated kDNA, DCT.

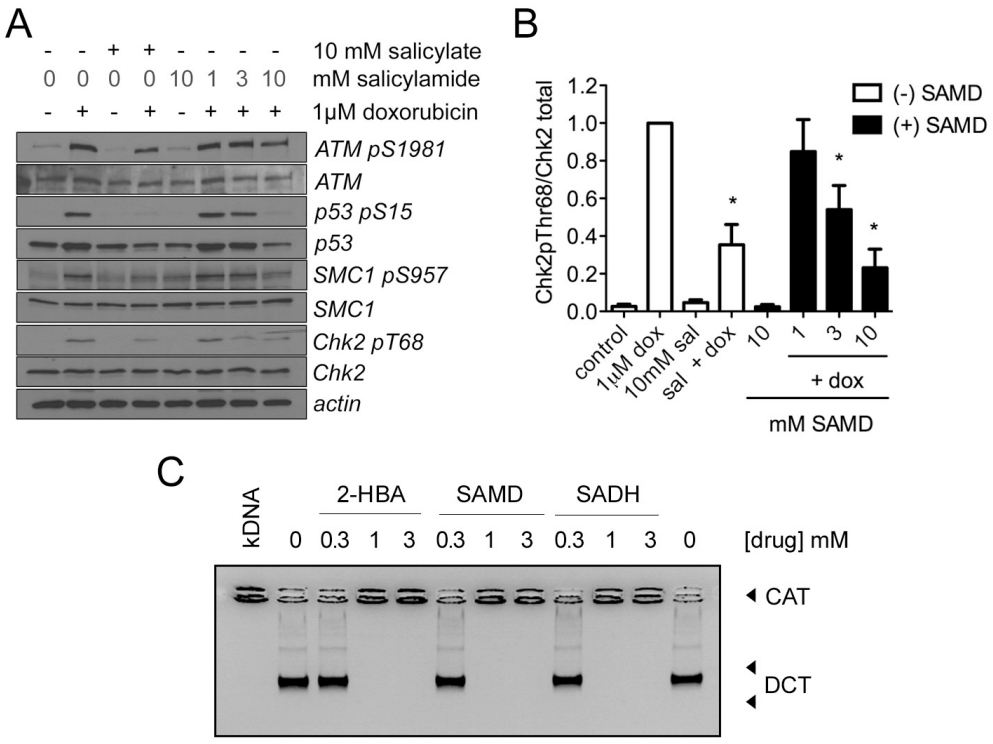

A
- - + + - - - - 10 mM salicylate
0 0 0 0 10 1 3 10 mM salicylamide
- + - + - + + + 1μM doxorubicin
ATM pS1981
ATM
p53 pS15
p53
SMC1 pS957
SMC1
Chk2 pT68
Chk2
actin
B
Chk2pThr68/Chk2 total
1.2
1.0
0.8
0.6
0.4
0.2
0.0
(-) SAMD
(+) SAMD
control
1μM dox
10mM sal
sal + dox
10
1
3
10
+ dox
mM SAMD
C
kDNA
2-HBA
SAMD
SADH
0 0.3 1 3 0.3 1 3 0.3 1 3 0
[drug] mM
CAT
DCT

Figure 5.7. Modification of the carboxylate moiety does not alter the ability to inhibit topoisomerase II.

Modification of the carboxylate moiety was examined through the use of salicylamide and salicylaldehyde. (A, B) MCF-7 cells were treated with salicylate (sal; 10 mM) or increasing concentrations of salicylamide (SAMD) for 1 h prior to the addition of doxorubicin (dox; 1 μM) and continued incubation for an additional 2 h. Whole cell extracts were prepared, immunoblotted and analyzed as in Fig. 5.3. The effects of SAMD were examined in three independent replicate experiments; representative immunoblots are shown. The standard error bars represent the S.E.M. Statistical significance of pretreatment effects compared with doxorubicin alone was determined by one-way ANOVA with a Tukey post hoc test (*; $p < 0.05$). (C) The ability of salicylate (2-HBA), SAMD or salicylaldehyde (SADH) to inhibit topo II catalytic activity *in vitro* was examined using catenated kDNA as a substrate. Reaction products were resolved by agarose gel electrophoresis and stained with ethidium bromide. A gel representative of three independent experimental replicates is shown. Catenated kDNA, CAT; decatenated kDNA, DCT.

signaling. Nevertheless, the compound was able to effectively inhibit topo II catalytic activity *in vitro* (Fig. 5.7C).

5.4.5 Clinically used salicylates, sulfasalazine and diflunisal, are effective inhibitors of topoisomerase II catalytic activity

While ASA may be the most well-known and widely consumed salicylate-based drug, a number of other salicylate derivatives are clinically used, including sulfasalazine and diflunisal. Both compounds retain a 2'-hydroxyl moiety and bear larger substituents at the 5'-ring position. Pretreatment of MCF-7 cells with either diflunisal (Figs. 5.8A and B) or sulfasalazine (Figs. 5.8C and D) led to more potent attenuation of doxorubicin-induced DNA damage signaling than observed with salicylate alone. In both cases, the concentrations required for attenuation were comparable to those observed with 2,3,4-THBA. The effects seen with diflunisal and sulfasalazine suggest that additions at the 5'-position can significantly enhance the ability of these compounds to attenuate the DNA damage response activated by topo II poisoning. We subsequently examined the effects of diflunisal and sulfasalazine, along with 2,5-DHBA, on topo II decatenation activity *in vitro* and observed that all were more potent than salicylate at inhibiting topo II catalytic activity (Fig. 5.8E), further supporting a role for modifications at the 5'-ring position.

The clinical effects of sulfasalazine are often attributed to its two metabolites: sulfapyridine and 5-ASA. Thus, we tested whether either of these metabolites altered doxorubicin-induced DNA damage signaling. Surprisingly, neither sulfapyridine nor 5-ASA modulated doxorubicin-induced DNA damage signaling, even at concentrations above those used for sulfasalazine (Fig. 5.8F).

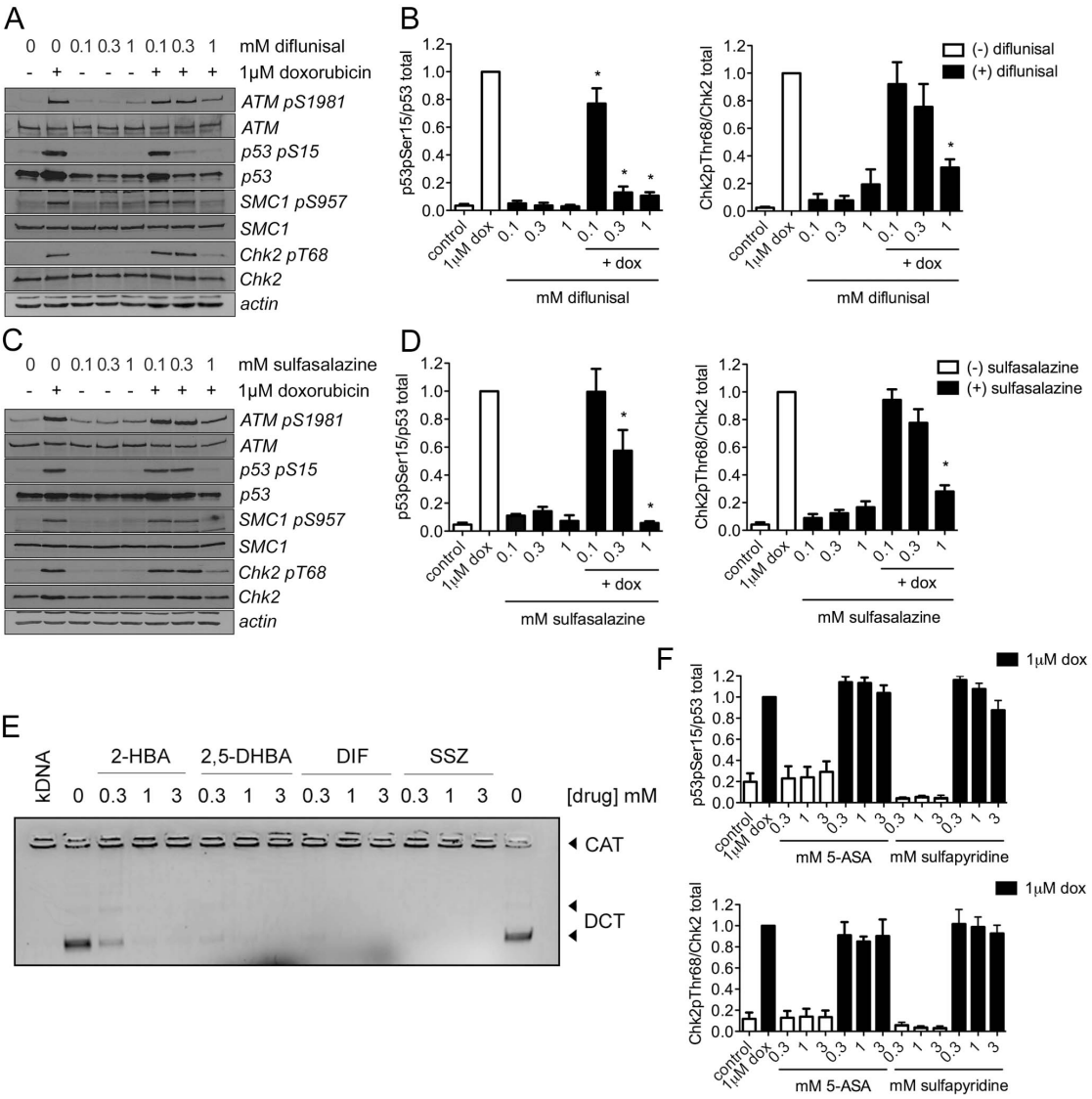

A
0 0 0.1 0.3 1 0.1 0.3 1 mM diflunisal
- + - - - + + + 1μM doxorubicin
ATM pS1981
ATM
p53 pS15
p53
SMC1 pS957
SMC1
Chk2 pT68
Chk2
actin
B
p53pSer15/p53 total
control
1μM dox
0.1 0.3 1 0.1 0.3 1
+ dox
mM diflunisal
Chk2pThr68/Chk2 total
(-) diflunisal
(+) diflunisal
C
0 0 0.1 0.3 1 0.1 0.3 1 mM sulfasalazine
- + - - - + + + 1μM doxorubicin
D
mM sulfasalazine
(-) sulfasalazine
(+) sulfasalazine
E
kDNA
2-HBA
2,5-DHBA
DIF
SSZ
0 0.3 1 3 0.3 1 3 0.3 1 3 0.3 1 3 0 [drug] mM
CAT
DCT
F
1μM dox
mM 5-ASA
mM sulfapyridine

Figure 5.8. Diflunisal and sulfasalazine are catalytic inhibitors of topoisomerase II.

MCF-7 cells were treated with increasing concentrations of diflunisal (n=5; A, B) or sulfasalazine (n=3; C, D) for 1 h prior to the addition of doxorubicin (dox; 1 μM) and continued incubation for an additional 2 h. Whole cell extracts were prepared, immunoblotted and analyzed as in Fig. 5.3. Representative immunoblots are shown for each compound. The error bars represent the S.E.M. Statistical significance of pretreatment effects compared with doxorubicin alone was determined by one-way ANOVA with a Tukey post-hoc test and is denoted by an asterisk (*; $p < 0.05$). (E) The effects of salicylate (2-HBA), 2,5-dihydroxybenzoic acid (2,5-DHBA), diflunisal (DIF) and sulfasalazine (SSZ) on topo II decatenation activity were examined using catenated kDNA as a substrate. Reaction products were resolved by agarose gel electrophoresis and stained with ethidium bromide. A gel representative of at least three independent experimental replicates is shown. Catenated kDNA, CAT; decatenated kDNA, DCT. (F) The metabolites of sulfasalazine, 5-aminosalicylic acid (5-ASA; n=3) and sulfapyridine (n=3), were tested for their ability to attenuate doxorubicin-induced DNA damage signaling. The blots of phosphorylated and total pools of Chk2 from three independent replicates for each compound were scanned, analysed densitometrically and plotted. The error bars represent the S.E.M. Statistical significance of pretreatment effects compared with doxorubicin alone was determined by one-way ANOVA with a Tukey post-hoc test. No statistically significant effects were observed with 5-ASA or sulfapyridine.

5.4.6 Sulfasalazine and diflunisal inhibit topoisomerase II ATPase activity and prevent topo II-mediated DNA cleavage

In a recent study, we determined that salicylate attenuates doxorubicin-induced DNA damage signaling by blocking topo II-DNA cleavage, thereby preventing the formation of doxorubicin-stabilized covalent topo II-DNA complexes (Bau and Kurz, 2011; Bau et al., 2014). To examine if treatment of cells with either sulfasalazine or diflunisal prevents the formation of doxorubicin-stabilized cleavable complexes in intact cells, we used the ICE bioassay (Bau and Kurz, 2011; Subramanian et al., 2001). In a manner similar to salicylate, pretreatment of MCF-7 cells with either diflunisal or sulfasalazine blocked the formation of doxorubicin-induced topo II-DNA cleavable complexes in cells (Fig. 5.9A).

To compare the mechanism by which diflunisal and sulfasalazine inhibit topo II catalytic activity, we examined their effects on topo II ATPase activity and DNA cleavage as we previously had done for salicylate (Bau et al., 2014). Using [γ-^{32}P]-ATP and purified topo IIα, we determined that both diflunisal and sulfasalazine inhibit topo II-mediated ATP hydrolysis in a dose-dependent manner (Figs. 5.9B and C). The concentrations of sulfasalazine and diflunisal required for inhibition are at least 3-fold lower than that required for salicylate in this assay (Bau et al., 2014). We then examined the effects of sulfasalazine and diflunisal on topo II-mediated DNA cleavage using purified topo IIα. Topo II establishes two cleavage equilibria: the first prior to strand passage (ATP-independent), and the second immediately after strand passage (ATP-dependent). In the presence of salicylate, we previously determined that both cleavage events are inhibited (Bau et al., 2014); however, DNA relegation was not affected by salicylate. To determine if sulfasalazine or diflunisal inhibit topo II-mediated DNA cleavage, we examined DNA

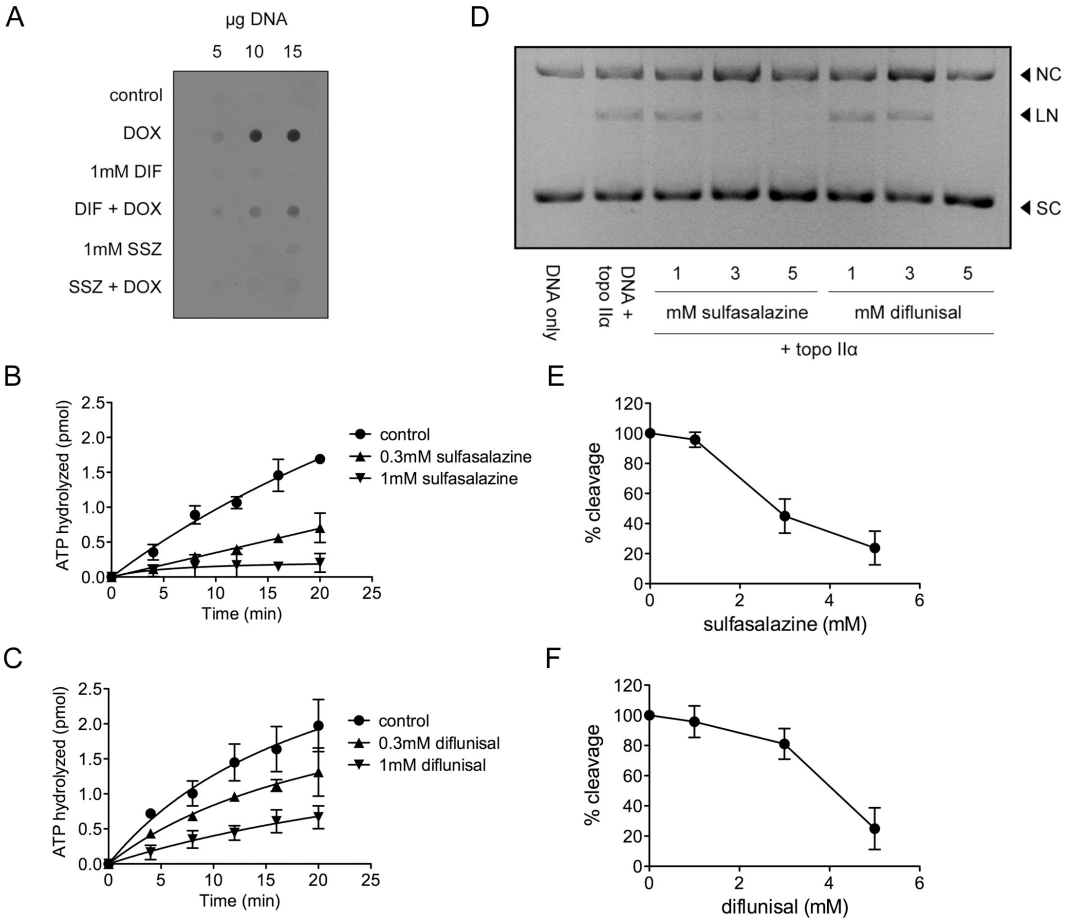

A
μg DNA
5 10 15
control
DOX
1mM DIF
DIF + DOX
1mM SSZ
SSZ + DOX
D
NC
LN
SC
DNA only
DNA + topo IIα
1 3 5 1 3 5
mM sulfasalazine
mM diflunisal
+ topo IIα
B
ATP hydrolyzed (pmol)
Time (min)
control
0.3mM sulfasalazine
1mM sulfasalazine
E
% cleavage
sulfasalazine (mM)
C
ATP hydrolyzed (pmol)
Time (min)
control
0.3mM diflunisal
1mM diflunisal
F
% cleavage
diflunisal (mM)

Figure 5.9. Sulfasalazine and diflunisal are catalytic inhibitors of topoisomerase II that block with DNA cleavage.

(A) Formation of topo II-DNA cleavable complexes in MCF-7 cells was monitored using the *in vivo* complex of enzyme (ICE) bioassay. Cells were treated with either diflunisal (DIF; 1 mM) or sulfasalazine (SSZ; 1 mM) for 1 h prior to the addition of doxorubicin (DOX; 1μM) and continued incubation for an additional 2 h. Cells were lysed rapidly and extracts were partitioned through a cesium chloride gradient. The fraction containing DNA was collected, sonicated, quantified and spotted onto a nitrocellulose membrane. The membrane was then probed for DNA-bound topo II using an antiserum specific for human topo IIα. An immunoblot representative of three independent experimental replicates is shown. Topo II-mediated ATP hydrolysis was measured by TLC in the presence of sulfasalazine (B) or diflunisal (C). Untreated control samples (control) were collected alongside sulfasalazine- and diflunisal-treated samples. The means and S.E.M. of three independent experiments for each compound are plotted. (D) Topo II-mediated cleavage of plasmid DNA was carried out in the absence of ATP and the presence of Ca2+. DNA was incubated with topo IIα (10 U) for 15 min at 37°C in the presence of diflunisal or sulfasalazine. Reactions were stopped by the addition of SDS prior to digestion with proteinase K. Plasmid DNA was ethanol precipitated, resuspended and resolved on a 1% agarose gel containing 0.7 mg mL-1 ethidium bromide for 15 h at 12V. A gel representative of five independent experimental replicates is shown. SC, supercoiled DNA; LN, linear DNA; NC, nicked circular DNA. (E, F) The bands corresponding to linearized pBR322 in (D) were analyzed by densitometry and intensities were averaged and normalized to DNA with topo II alone. The graphs represent the means and S.E.M. of five independent experimental replicates for each drug.

cleavage in the presence of Ca^{2+} and the absence of ATP. This permits DNA cleavage but prevents strand passage. In the presence of either sulfasalazine or diflunisal, topo II-mediated DNA cleavage was completely blocked, with sulfasalazine being modestly more potent than diflunisal at inhibiting enzyme-mediated cleavage (Figs. 5.9D-F). The concentrations at which this was observed were approximately 10-fold lower than that required for salicylate-mediated inhibition of DNA cleavage (Bau et al., 2014).

5.5 Discussion

Topo II is essential for modulating DNA topology, a process that is tightly controlled to allow for DNA replication, transcription and cell division (Nitiss, 2009a; Vos et al., 2011). The targeting of topo II by poisons is widely exploited in cancer chemotherapy as these agents stabilize the topo II-DNA cleavable complex, generating DNA double-stranded breaks (Nitiss, 2009b; Pommier, 2013; Pommier et al., 2010); however, these poisons target the catalytic cycle at only one step, while catalytic inhibitors can block enzyme activity at one of the other steps in the catalytic cycle without generating DNA double-stranded breaks (Larsen et al., 2003).

We previously identified salicylate as a topo II catalytic inhibitor (Bau and Kurz, 2011) that prevents DNA cleavage and is selective for the alpha isoform of topo II (Bau et al., 2014). As a secondary consequence of inhibiting strand passage, salicylate also decreases topo II ATPase activity (Bau et al., 2014). Pretreatment of cells with salicylate prior to the addition of doxorubicin prevents topo II-DNA cleavable complex formation, leading to decreased doxorubicin cytotoxicity (Bau and Kurz, 2011). It has been proposed that salicylate (and likely structurally-related compounds) interacts with the minor groove of DNA, thereby distorting the helical structure (Bathaie et al., 2010). As topo II must bind

DNA before initiating DNA cleavage, any compound that distorts the DNA structure would also lead to a concomitant loss of DNA cleavage. However, our previous work using multiple biochemical assays demonstrates that salicylate does not impair topo II-DNA binding (Bau et al., 2014). Given that salicylate-related compounds are found both naturally occurring in foods, as well in a number of commonly used pharmaceuticals, we sought to investigate whether other salicylate-related agents also inhibited topo II.

We evaluated these compounds for their ability to decrease doxorubicin-induced DNA damage signaling. We have previously demonstrated that pretreatment of cells with salicylate attenuates this response, observed as a decrease in the phosphorylation of ATM and its downstream effectors (Bau and Kurz, 2011). This response is dose-dependent and provides an initial assessment for the comparison of salicylate and its congeners. Although benzoate and salicylate differ by only a single hydroxyl group, this produces a marked shift in potency when inhibiting topo II activity *in vitro* (Fig. 5.1). Thus, we asked whether the number and placement hydroxyl groups alters the concentration of these agents required to attenuate doxorubicin-induced DNA damage signaling. A number of flavonoid glycosides have been shown to inhibit topo II (Tselepi et al., 2011) and the number of hydroxyl moieties has proposed to dictate the potency of these compounds (Bandele et al., 2008). For compounds bearing a single hydroxyl group, position did not alter the degree of attenuation of doxorubicin-induced DNA damage signaling (Fig. 5.3), but transfer of the hydroxyl group to the 3'- or 4'-position was associated with less effective inhibition of topo II decatenation activity *in vitro* when compared with salicylate (Fig. 5.5E). This finding suggests that, at least *in vitro*, the 2'-position of salicylate plays an important role in mediating potency of topo II inhibition.

Addition of a second hydroxyl substituent did not markedly impact the attenuation of doxorubicin-induced DNA damage signaling when compared with salicylate (Fig. 5.4). However, when we tested these compounds in a cell-free system, we were able to discern differences in their relative ability to inhibit topo II. The most striking effect was observed with the addition of a hydroxyl group at the 5'-position; although 2,5-DHBA and 3,5-DHBA were both more potent than salicylate, more importantly each compound was at least three-fold more potent at inhibiting decatenation than its respective single hydroxyl substituent, 2-HBA (salicylate) and 3-HBA (Figs. 5.5E and F). Taken together, these findings highlight the importance of the 5'-position, in addition to the 2'-position, in enhancing potency of topo II inhibition. Of note, 3,4-DHBA has previously been shown to reduce doxorubicin-mediated cytotoxicity, although the mechanism underlying this observation was not described (De Graff et al., 2003); our work now demonstrates that this is likely through direct topo II catalytic inhibition (Fig. 5.5E).

The addition of a third hydroxyl group (2,3,4-THBA and 3,4,5-THBA) resulted in enhanced inhibition of topo II catalytic activity, but divergent effects in the attenuation of doxorubicin-induced DNA damage signaling. While 2,3,4-THBA was the most potent compound tested for attenuating doxorubicin-induced DNA damage signaling, in contrast 3,4,5-THBA did not (Figs. 5.5A-D). In fact, at concentrations above 1 mM, 3,4,5-THBA activated an ATM-dependent signaling response, even in the absence of treatment with doxorubicin (data not shown). 3,4,5-THBA (also referred to as gallic acid) has previously been identified as a topoisomerase poison, targeting both topo I and topo II (López-Lázaro et al., 2011). Moreover, 3,4,5-THBA is a substituent of another potent topoisomerase poison, epigallocathechin-3-gallate, a compound found in high concentrations in green tea extracts (Bandele and Osheroff, 2008, 2009). The ability to poison topo II is consistent with

the triggering of DNA damage signaling we observed in cells treated with higher concentrations of 3,4,5-THBA. The inhibition of topo II decatenation that we observed with 3,4,5-THBA (Fig. 5.5F) is consistent with its ability to poison topo II, as the kDNA assay does not differentiate between catalytic inhibitors and poisons.

Our experiments with hydroxylated salicylate derivatives support a role for the 2'- and 5'-position in modulating potency of topo II inhibition. We examined compounds with alternate modifications at these sites to determine the nature of the substitutions required, beginning with substitutions at the 2'-position. Our data demonstrate that substitution with a hydrogen molecule (i.e. benzoate) is associated with a decrease in the potency of topo II inhibition (Figs. 5.1 and 5.5E) and decreased attenuation of doxorubicin-induced DNA damage signaling (Bau and Kurz, 2011). Replacement of the 2'-hydroxyl group with fluorine (2-FBA), which has an even smaller atomic radius than the hydrogen in benzoate, was associated with intermediate potency, particularly evident in the topo II decatenation assay (Fig. 5.6G). We then investigated the effects of larger substitutions at the 2'-position. We noted that replacement of the 2'-hydroxyl group with either a methyl or acetyl group was also associated with decreased attenuation of doxorubicin-induced DNA damage signaling (Figs. 5.6C-F). This was most evident at lower concentrations ($\leq$ 3mM) when compared with salicylate (Figs. 5.3A and D). These effects were also observed *in vitro*; at the concentrations tested, little inhibition of topo II decatenation activity was observed (Fig. 5.6G). Taken together, these data indicate that changes in substituent size alone, at the 2'-position, cannot account for the observed differences in potency of topo II inhibition.

Beyond their differences in size, the 2'-modified compounds we tested substitutions that differ in their electronegativity. Salicylate contains a hydroxyl group, which is an effective hydrogen bond donor by virtue of its electronegative oxygen atom. By

comparison, benzoate, 2-MBA and ASA possess terminal carbon-hydrogen bonds that are comparatively non-polar, making hydrogen-bond interactions with these compounds unlikely, although the oxygen atom in the acetyl group of ASA is capable of acting as a hydrogen bond acceptor. The carbon-fluorine bond found in 2-FBA is highly polar, owing to the electronegativity of fluorine. However, carbon-fluorine bonds only form weak hydrogen bonds while acting as hydrogen bond acceptors (Bissantz et al., 2010; Dunitz and Taylor, 1997). Given the relative potency of the compounds tested (2-HBA>2-FBA>ASA>2-MBA>benzoate), we postulate that effective topo II inhibition is observed when an electronegative hydrogen bond donor is present the at the 2'-position.

We also examined modifications to both the 5'-ring position and the carboxylate moiety of salicylate. Alterations to the carboxylate moiety did not alter the degree of topo II inhibition when compared with salicylate (Fig. 5.7). In contrast, addition of a hydroxyl group at the 5'-position enhanced topo II inhibition, which was not the case with placement of the second hydroxyl group in any other ring position (Figs. 5.5E and F). The importance of the 5'-position was further revealed in experiments using sulfasalazine and diflunisal; these clinically used agents contain 2'-hydroxyl groups in addition to larger substitutions at the 5'-position. With each of these compounds, we observed a striking increase in the potency of attenuation of doxorubicin-induced DNA damage signaling and inhibition of topo II decatenation activity when compared with salicylate (Fig. 5.8). We demonstrated that both sulfasalazine and diflunisal impair formation of doxorubicin-stabilized topo II cleavable complexes *in vivo* (Fig. 5.9A). Furthermore, we determined that they do so through inhibition of topo II-mediated ATP hydrolysis secondary to inhibition of topo II-DNA cleavage (Figs. 5.9B-F). Given the increased potency observed with 2,5-DHBA, diflunisal and sulfasalazine, substitution at the 5'-position clearly enhances the inhibition of

topo II catalytic activity. Investigations into the nature of substitutions at this position remain ongoing.

In this study, we systematically evaluated compounds with modifications on both the benzoate ring and its carboxylate moiety, allowing us to identify substitutions modulating the potency of topo II inhibition. In particular, our work supports a model that highlights the importance of hydrogen bond donating, electronegative substituents at the 2'-position in contributing to the potency of the catalytic inhibition of topo II and substitutions at the 5'-position for enhancing these effects (Fig. 5.10). Following upon our initial identification of salicylate as a novel catalytic inhibitor of human topo II (Bau and Kurz, 2011; Bau et al., 2014), our current work identifies a broader class of topo II catalytic inhibitors based on the structure of salicylate. Importantly, this includes several clinically used pharmaceuticals. Salicylate is the major metabolite of ASA, generated *in vivo* after rapid spontaneous hydrolysis or after transfer of the acetyl group to the catalytic serine residue of the cyclooxygenase enzymes (Lecomte et al., 1994; Mitchell et al., 1993). Clinical studies have determined that individuals taking high dose aspirin for reach plasma salicylate concentrations between 1 to 3 mM (Day et al., 1989; Hundal et al., 2002); thus, the salicylate concentrations at which topo II catalytic inhibition is observed are achievable. In addition, it has been observed that salicylate can reach millimolar concentrations in xenograft tumor sites in athymic mice following oral administration of a single low dose of ASA (equivalent to human consumption of a single 325 mg tablet) (Stark et al., 2007). Our studies indicate the ASA treatment of cells in culture and purified topo II *in vitro* is less effective than salicylate at attenuating doxorubicin-induced DNA damage signaling and inhibiting topo II catalytic activity. We postulate that this is due to the fact that ASA remains unhydrolyzed as it has previously been reported that hydrolysis under experimental

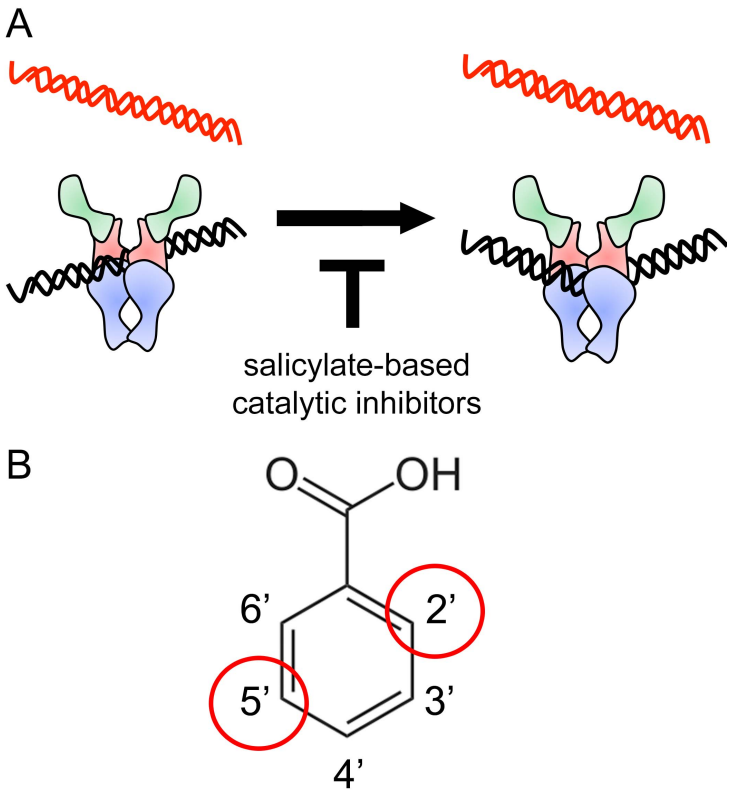


Figure 5.10. Model for inhibition of topoisomerase II catalytic activity by salicylate-related compounds.

Salicylate-related compounds, including sulfasalazine and diflunisal, inhibit topo II-mediated DNA cleavage. The potency of salicylate-related catalytic inhibitors appears to be strongly influenced by placement of electronegative substituents capable of hydrogen bonding at the 2'-position and larger, potentially anchoring, substituents at the 5'-position (red circles).

conditions similar to ours occur very slowly, in the order of days, if not weeks (James, 1958; Rainsford, 2004). Thus, although we observed limited effects of ASA in our experiments, it is expected that ASA would be rapidly hydrolyzed *in vivo*, liberating salicylate to inhibit topo II catalytic activity.

Aside from pharmaceutical sources of salicylate, many of the salicylate derivatives examined in this study are found in plant-based foods (Duthie and Wood, 2011; Paterson et al., 2006), as salicylic acid is a naturally occurring plant hormone involved in stress responses and host defense (Raskin, 1992; Vlot et al., 2009). Thus, salicylates can be ingested from a variety of sources leading to increased plasma levels of salicylate and related agents (Blacklock et al., 2001). We have previously demonstrated that salicylate, and now a wide variety of congeners, can attenuate the action of topo II poisons. This potentially poses a significant risk to individuals receiving topo II-targeting poisons as part of anti-cancer therapy. Although the experimental design used in this study was focused on the short-term effects of an acute exposure to salicylate, future studies aimed at examining the effects of long-term, low dose salicylate administration are warranted. Given that topo II poisons, like many cytotoxic chemotherapeutics, have narrow therapeutics indices, it is possible that co-administration with salicylates, even at low doses, may negatively impact chemotherapeutic efficacy. Certainly, further studies are required to assess the impact of these compounds and investigations in mouse models are currently underway in our laboratory.

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Chapter Six: General Discussion and Insights

6.1 Summary

Salicylates and related compounds are commonly used pharmaceuticals and are found ubiquitously in plant-based foods. The first, albeit indirect, use of salicylate was recorded in the Ancient era, when willow bark extracts were used for pain relief (Rainsford, 2004). The widespread use of salicylates began with the synthesis of aspirin (of which salicylate is the primary metabolite), becoming the first commercial drug marketed by the pharmaceutical giant Bayer in 1899. Today, salicylate and its derivatives are used to treat a variety of medical conditions characterized by pain and inflammation as well as for the prevention of secondary vascular events. At a cellular level, salicylates are known to affect numerous signaling pathways associated with inflammation, growth and proliferation. These collective findings have yielded great insight into bridging their function at a molecular level to their pharmacological effects at a systemic level.

In this thesis, a novel property of salicylate, of both biological and functional significance, was identified and characterized. This work demonstrates that salicylate and several structurally-related compounds are effective catalytic inhibitors of topo II. This work initially developed as a follow-up to an early observation that salicylate dose-dependently attenuates doxorubicin-induced DNA damage signaling in a variety of human cell lines. This observation subsequently evolved to work in human breast cancer cells establishing that salicylate is a novel catalytic inhibitor of topo II (Bau and Kurz, 2011; Chapter 3).

Salicylate has several described properties, one of which is the ability to scavenge reactive oxygen species. As such, the initial hypothesis for this project was that the

observed attenuation of doxorubicin-induced DNA damage was attributable to the scavenging of hydroxyl radicals, generated as a byproduct of redox cycling of doxorubicin through the Fenton reaction. However, early studies demonstrated that salicylate attenuates DNA damage signaling induced by other topo II poisons, including those incapable of redox cycling, such as 5-iminodaunorubicin, etoposide and mitoxantrone. Thus, it was concluded that the attenuation of topo II poison-induced DNA damage signaling was unlikely due to hydroxyl radical scavenging.

Doxorubicin poisons topo II by trapping the enzyme in a covalent complex with DNA, the removal of which exposes of a DNA double-stranded break. The observed attenuation of DNA damage signaling by salicylate is associated with a concomitant loss in doxorubicin-induced DNA double-stranded breaks. When it was examined whether stabilization of doxorubicin-induced topo II cleavable complexes was indeed blocked by pretreatment with salicylate, a substantial abrogation of these protein-DNA adducts was observed. Perhaps most significantly, the loss of DNA strand breaks with salicylate pretreatment leads to reduced doxorubicin-mediated cytotoxicity. Considered together, these findings indicate that salicylate mirrors properties of established topo II catalytic inhibitors. In keeping, incubation of recombinant purified topo II with salicylate was shown to inhibit catalytic activity without stabilization of the enzyme as a covalent adduct on DNA, indeed indicating that salicylate is a *bona fide* catalytic inhibitor of topo II.

As many of the studies examining the pharmacological mechanisms of salicylate utilize the compound at concentrations similar to those used in this work, great care to control for these alternate mechanisms was exercised. The experiments demonstrate with confidence that salicylate is a direct catalytic inhibitor of topo II, and that the effects on DNA damage signaling observed likely are not the result of non-specific attenuation of

other salicylate targets, such as IKK or NFκB signaling, or the inhibition of cyclooxygenases. In doing so, a novel target for a long-standing, even ancient, drug has been identified.

Topo II catalytic inhibitors differ from topo II poisons; catalytic inhibitors do not trap topo II in a covalent cleavable complex with DNA (Larsen et al., 2003). Rather, catalytic inhibitors interfere with enzyme activity at stages of the catalytic cycle other than cleavable complex formation, including DNA binding, DNA cleavage, ATPase activity and enzyme dissociation. Identifying the precise mechanism of topo II inhibition by salicylate became the focus of the second part of this thesis work (Chapter 4). Each step of the catalytic cycle was systematically interrogated using one or more biochemical assays to examine the effect of salicylate *in vitro* (Bau et al., 2014; Chapter 4). Salicylate was found to have no effect on DNA binding, DNA religation or closed clamp formation. However, as expected with compounds that interfere with catalytic activity, addition of salicylate led to a decrease in topo II-mediated ATP hydrolysis. This was in a manner consistent with a non-competitive mode of inhibition, indicating that salicylate does not directly compete with the ATP binding site, located in the N-terminal domain of the enzyme. The promiscuity of salicylate for ATPase inhibition was examined by testing its effects on apyrase, a general E-type ATPase; however, no salicylate-mediated inhibition of apyrase was observed. Using assays to examine both pre-strand passage and post-strand passage DNA cleavage, it was observed that salicylate blocks DNA cleavage, disrupting both equilibria. Additional studies then examined the effects of salicylate on each of the human isoforms, topo IIα and topo IIβ. Surprisingly, experiments using equal units of each isoform demonstrated that salicylate selectively inhibits the alpha isoform, making salicylate only the second known topo IIα selective catalytic inhibitor.

In early experiments addressing the antioxidant hypothesis, it was noted that benzoate was ten-fold less potent at attenuating doxorubicin-induced DNA damage signaling than salicylate, despite the fact that they differ by only a single hydroxyl group at the 2'-position. This finding suggests that discrete chemical modifications to salicylate influence the potency of topo II inhibition. Many pharmaceuticals and compounds found naturally in foods closely resemble salicylate in structure. Naturally occurring derivatives of salicylate, such as the hydroxybenzoic acids, are found in plants and salicylate is well characterized as a plant hormone involved in the stress response. To examine structural determinants contributing to topo II inhibition, derivatives of salicylate were systematically evaluated (Chapter 5). Many of the compounds tested inhibited topo II activity *in vitro* and attenuated doxorubicin-induced DNA damage signaling with varying levels of effectiveness. Hydroxyl group placement and substitutions at the 2'- and 5'- positions most strongly influenced the potency of topo II inhibition *in vitro*. Of the clinically used salicylates, both sulfasalazine and diflunisal (compounds with both 2'- and 5'- substitutions) were effective at inhibiting enzyme activity and blocking topo II DNA cleavage, whereas aspirin (bearing a blocked 2'- position and lacking a 5'- substituent) was only weakly effective *in vitro*. In keeping with the requirement for a salicylate core structure, examination of non-salicylate-based non-steroidal anti-inflammatory drugs of multiple structural classes failed to identify any compound capable of attenuating doxorubicin-induced DNA damage signaling (Appendix 1).

6.2 Implications of salicylates as novel catalytic inhibitors of topoisomerase II

Salicylates are a widely studied class of drugs having a multitude of cellular effects. While these effects are commonly associated with attenuating inflammatory responses,

salicylate has also been demonstrated to inhibit cell growth by promoting apoptosis (Baviskar et al., 2011; Elder et al., 1996; Law et al., 2000). More recent studies have shown that salicylate activates AMP kinase, a key effector in glucose regulation (Hawley et al., 2012). The findings described in this thesis expand on the growing body of literature defining the biochemical properties of salicylate and describe salicylate as a novel catalytic inhibitor of topo IIα.

These novel findings may also help explain earlier observations noted with salicylates. For example, as topo IIα is critical for cell division, one might hypothesize that the growth inhibition observed with salicylate treatment maybe directly related to topo IIα inhibition. It has been observed that topo II inhibition leads to apoptotic cell death, a finding that has also been noted with salicylate. Once again, it could be postulated that salicylate-mediated apoptosis may partially be impacted by the ability of the compound to impair topo II activity. While salicylate has many cellular targets, one could interrogate this by selective knockdown of topo IIα using siRNA and determining whether this promotes resistance to salicylate-mediated cell death. A similar approach was utilized in a recently published study studying the selectivity of the topo IIα selective catalytic inhibitor vescalagin (Auzanneau et al., 2012). Cells with decreased topo IIα levels were partially resistant to vescalagin treatment, and as our data suggest selectivity of salicylate for the α isoform, we might hypothesize a similar outcome.

On a broader scale, the identification of salicylate as a novel isoform-selective catalytic inhibitor of topo II sets the foundation for future work aimed at identifying more potent selective inhibitors of topo II. As a cleavage-interrupting inhibitor, the salicylates identified in this work significantly expand the number of compounds known to inhibit DNA cleavage, a mechanism previously characterized for merbarone (Fortune and

Osheroff, 1998). However, in contrast to merbarone, which is also known to induce DNA damage (Attia et al., 2003; Pastor et al., 2012), salicylate does not appear to do so.

Salicylate preferentially inhibits the topo IIα isoform. Topo IIα is critical in DNA replication, and is heavily implicated in determining tumor sensitivity to topo II poisons (O'Malley et al., 2009; Pritchard et al., 2008). In contrast, topo IIβ has roles in neural development and regulated transcription, and is expressed in both proliferating and post-mitotic cells (Ju et al., 2006; Lyu and Wang, 2003). Importantly, it is the poisoning of topo IIβ that is implicated in the significant long-term side effects associated with topo II targeting chemotherapeutics (Cowell et al., 2012; Zhang et al., 2012). Given that most topo II poisons non-selectively target both the alpha and beta isoforms, there is clinical significance for either the identification of novel topo IIα-selective poisons or in the identification of tissue protective topo IIβ-selective catalytic inhibitors. Further development of this research area is critical if chemotherapeutic regimens are to be made safer, and to avoid the significant side effects associated with non-specific poisoning of the topo IIβ isoform. Despite having virtually identical catalytic mechanisms and considerable sequence homology between the two isoforms, development of isoform-selective therapeutics may prove to be a challenging task. Encouragingly, however, the work in this thesis indicates that even compounds as structurally simple as salicylate can distinguish between the two isoforms.

To our knowledge, salicylate is only the second known catalytic inhibitor demonstrating isoform selectivity, the first being vescalagin, a compound that also targets the topo IIα isoform (Auzanneau et al., 2012). In addition to blocking topo IIα activity *in vitro,* vescalagin also blocks the formation of etoposide-induced topo IIα, but not topo IIβ, covalent complexes, in keeping with its classification as a catalytic inhibitor.

In an effort to identify the structural determinants modulated salicylate-mediated catalytic inhibition of topo II, derivatives of salicylate were examined to determine if any 'patterns' could be identified. Modifications based on the both the nature and placement of substituents were examined. Indeed, discrete changes, such as increasing the number of hydroxyl groups or placing substituents on the 2'- or 5'- position of benzoate, markedly influenced the ability of a compound to inhibit topo II. These findings have revealed potential factors influencing potency. For example, the ability of the 2'-moiety to hydrogen bond appears to be a marked determinant of topo II inhibition. As the hydroxyl group is a known hydrogen bond donor, its presence at the 2'-position might suggest that the orientation of the compound when in complex with topo II is an important factor in inhibiting enzyme activity. Depending on where salicylate binds on topo II, certain amino acid residues may be more likely to form hydrogen bond interactions with salicylate. For example, several amino acid groups that are capable of hydrogen bonding, including aspartic acid or glutamic acid, are important in coordinating the topo II-DNA cleavage reaction. As salicylate blocks DNA cleavage, salicylate may interfere with the enzyme-DNA transition state, leading to the observations presented in this thesis. This may also explain why, at least *in vitro*, an increase in the number of hydroxyl groups is associated with an increase in potency, as the close proximity of these amino acid residues could increase the number of hydrogen bond interactions between the drug and topo II.

In addition to the role played by the 2'-position, the presence of a substituent at the 5'-position (especially larger groups as seen with sulfasalazine and diflunisal) indicate that this position might serve to anchor the compound in a drug binding pocket on topo II. Without additional studies using a medicinal chemistry approach, it is not possible to discern additional information on chemical properties at this position that influence

potency. However, the findings described in this work establish a foundation for using salicylate as a structural backbone to guide future catalytic inhibitor development.

6.3 Challenges in studying the biological mechanisms of salicylates

As with any scientific inquiry, there are limitations that must be considered when interpreting the experimental results. Caution must be exercised in the translation of findings with salicylates from cell-based models to *in vivo* settings. In the adult population, long-term salicylate use (such as with aspirin) is associated with increased risks of gastrointestinal irritation and prolonged bleeding time due to effects on platelet aggregation; salicylates are no longer recommended in pediatric populations due to the link with Reye's syndrome, a potentially life-threatening reaction. Clinical studies have determined that individuals taking high dose aspirin (approximately 6 to 7 grams per day) reach plasma salicylate concentrations between 1 to 3 mM, with only acute ototoxicity observed at these concentrations (Day et al., 1989; Hundal et al., 2002). Severe salicylate toxicity occurs at plasma concentrations exceeding 0.75 g L^{-1} (approximately 4.7 mM), although individuals reaching higher plasma concentrations have been reported (Galbois et al., 2009). For these combined reasons, the prolonged use of salicylates at high doses has fallen out of favor, with salicylate more commonly used as long-term low dose therapy for the prevention of secondary cardiovascular and cerebrovascular events. This raises caution regarding the interpretation of laboratory-based and preclinical studies that have commonly used salicylate concentrations between 5 and 10 mM. It is at these concentrations that the effects of salicylate become most prominent in pre-clinical studies, including its effects in regulating glucose metabolism and in inhibiting the NFκB pathway (Hawley et al., 2012; Hundal et al., 2002). With respect to the studies described in this thesis, partial attenuation

of doxorubicin-induced DNA damage signaling was observed in acute exposures at concentrations as low as 3 mM, but maximally at 10 mM. In *in vitro* assays using purified topo II, complete inhibition of catalytic activity was observed at 1 mM (Chapter 3). Thus, effects on the catalytic activity of topo II are observed at relatively high concentrations. However, the experimental design used in these studies was focused on the short-term effects of an acute exposure to salicylate. Such parameters would be unlikely to be found in patients and, thus, future studies aimed at examining the effects of long-term, low dose salicylate administration are warranted. This would require the examination of alternate endpoints. A possible study design, currently being examined, is whether multi-day low-dose salicylate treatment can affect doxorubicin efficacy in a murine model. To this end, it is being examined whether tumor formation and overall survival are negatively affected by salicylate co-administration. Given that topo II poisons, like many cytotoxic chemotherapeutics, have narrow therapeutic indices, it is possible that co-administration with salicylates, even at low doses, may negatively impact chemotherapeutic efficacy. Future work utilizing both cell-based and *in vivo* approaches are required to elucidate further the potential impact co-administration of these agents may have.

Sulfasalazine and diflunisal were both observed to be more potent than salicylate in cell-based treatments and in cell-free *in vitro* studies. While high dose salicylate would not be used clinically to catalytically inhibit topo II *in vivo*, the concentrations of sulfasalazine and diflunisal required for this effect are clinically achievable at therapeutic concentrations in human subjects. Both sulfasalazine and diflunisal are in clinical use as anti-inflammatory agents, although sulfasalazine more widely than diflunisal. Sulfasalazine has had prominent use in inflammatory bowel disease, Crohn's disease as well as rheumatoid arthritis (Kaufmann and Taubin, 1987; Suarez-Almazor et al., 2000). Diflunisal is

prescribed for chronic pain relating to osteoarthritis and rheumatoid arthritis (Wasey et al., 2010). Of concern, individuals regularly administered sulfasalazine (4 grams per day) can have plasma concentrations up to 100 μM (Klotz, 1985; Schröder and Campbell, 1972). Individuals receiving regular diflunisal treatment can reach as 300 μM (Nuernberg et al., 1991; Tobert et al., 1981). Thus, it is possible that patients with these conditions who are co-administered these salicylate-base NSAIDs in conjunction with topo II-targeting poisons could potentially be altering the chemotherapeutic efficacy of their treatments, especially given the narrow therapeutic window of these chemotherapeutics. While the use of either sulfasalazine or diflunisal is not widespread (compared to aspirin), in this context it is important to note that topo II catalytic inhibitors are more prevalent than first thought and will require further study to determine their impact current chemotherapeutic outcomes.

6.4 Directions for future studies

The findings from this research pave the way for multiple avenues of study that could significantly extend our understanding of the relationship between salicylates and topo II. The specificity of salicylate for the topo IIα isoform is an intriguing finding and could be used for further study elucidating the salicylate-binding site, which may then guide future studies as to why salicylate is selective for the topo IIα isoform. Expanded analysis of salicylate-related compounds may yield a better understanding of the chemical determinants modulating potency and, possibly, dictating isoform specificity. Perhaps most pressingly, studies in *in vivo* models of cancer would determine if salicylate and related compounds negatively influence chemotherapeutic efficacy of regimens incorporating topo II poisons. Ultimately, these future studies will work to build our understanding of the potential value or impact of salicylates as catalytic inhibitors of topo II.

6.4.1 Determining a binding site for salicylate and related compounds on topoisomerase II

To understand the relationship between topo II and salicylates, studies are required to identify the drug-binding site on topo II. Studies mapping the binding site of other compounds with topo II has yielded significant clues into both drug mechanism and enzyme function, the most notable examples being dexrazoxane and etoposide (Classen et al., 2003; Wu et al., 2011a). Identification of the dexrazoxane binding site expanded understanding of the function of the N-terminal protein clamps after trapping of the T-segment (Classen et al., 2003). Mapping of the etoposide binding site within the topo II cleavage complex has provided insight into potential mechanisms for drug resistance (Wu et al., 2011a).

As was determined through the experiments described in this thesis, salicylate interferes with DNA cleavage without impacting DNA binding. Unlike dexrazoxane, which binds in a newly formed pocket formed by the adjacent N-terminal clamps, salicylate does not promote formation of an enzyme-closed clamp (Bau et al., 2014). The precise mechanism contributing to the block in DNA cleavage remains unknown. Topo II-mediated DNA cleavage requires the presence of a divalent cation (specifically Mg^{2+}). Given that salicylate contains negatively charged substituents, the ionic bonds that form between the 2'-hydroxyl and carbonyl substituents and the Mg^{2+} ion could be speculated to interfere with DNA cleavage. A similar mechanism has been demonstrated for the antibacterial, DNA gyrase-targeting fluoroquinolones (Wohlkonig et al., 2010). These compounds poison DNA gyrase by disrupting the cation-DNA-enzyme transition state required for coordinating the DNA cleavage event. It may be that fluoroquinolones poison the enzyme due to their bulky size (in particular the presence of planar aromatic rings) whereas salicylate-based compounds block the cleavage reaction without trapping the

enzyme on DNA. Alternatively, as already discussed, salicylate might mask one of the amino acids required for DNA cleavage. Based on our findings in Chapter 5 with structural derivatives at the 2'-position that demonstrate the importance of hydrogen bond-donating properties, it is possible that the salicylate binds to aspartic acid and glutamic acid residues essential for cleavage coordination in the catalytic core of the enzyme (Deweese et al., 2008; Schmidt et al., 2010). In particular, if these residues are indeed important for enzyme inhibition by salicylate, one could generate topo II constructs with point mutations within the putative binding site. If these topo II mutants were to show resistance to salicylate-based inhibition, this would lend support to the hypothesis that hydrogen bond interactions between topo II and salicylate are critical for enzyme inhibition. However, if these topo II mutants do not show resistance towards salicylate, then a possibility that must be considered is that the binding site occurs outside of the core cleavage domain. Given our findings that salicylate is selective for the topo IIα isoform, and that this isoform shares a catalytic mechanism almost identical to the topo IIβ isoform, it is plausible that the salicylate binding site resides outside this cleavage domain.

In an attempt to identify the salicylate binding site on topo II, a hydrogen-deuterium exchange approach was undertaken in collaboration with the laboratory of Dr. David Schriemer (University of Calgary). This method maps shifts in surface, solvent accessible hydrogens (that can now be exchanged for deuterium) following drug binding. The deuterium-labeled protein is then digested and analyzed by mass spectrometry. Hydrogen-deuterium exchange is typically undertaken with smaller proteins. Nevertheless, using purified human topo IIα and AMPPNP, a reasonable coverage of the N-terminal three-quarters of the enzyme could be obtained and the ATP binding pocket was accurately mapped. When the topo II was incubated with salicylate, no binding site could be mapped.

The reason for this may be several-fold. Few peptides identified by mass spectrometry could be mapped to the C-terminus of the enzyme, the region that differs between the alpha and beta isoforms and that may contribute to the isoform specificity of salicylate. As salicylate prevents DNA cleavage, its interaction with topo II may require the presence of DNA. Unfortunately, the need for a mass spectrometric analysis in hydrogen-deuterium exchange precludes the inclusion of DNA in the binding reaction. Finally, salicylate is required at relatively high concentrations for inhibition of topo II. If the binding affinity of salicylate is too low, it may not be possible to map its binding site using hydrogen-deuterium exchange. Despite the challenges of using a hydrogen-deuterium exchange approach, identifying the binding site remains a worthwhile endeavor to better understand the interaction between the drug and enzyme, and clearly other approaches, such as protein crystallography, will have to be employed.

As an alternative approach to mapping regions important for salicylate-mediated inhibition of topo II, one could use a biochemical approach by generating topo II constructs in which regions of the topo IIα and topo IIβ isoform have been exchanged. A domain-swapping approach has previously been used to examine the function of the C-terminal domain (Gilroy and Austin, 2011). As previously stated, the greatest variance between the topo IIα and topo IIβ isoforms occurs in the C-terminal quarter of the enzyme. Thus, a likely, but not exclusive, hypothesis given our data demonstrating the isoform selectivity of salicylate is that a binding site resides within this region. Hybrid constructs could be purified from yeast and examined using *in vitro* decatenation assays to determine if swapping discrete domains alters salicylate-mediated inhibition of catalytic activity. Using this method, it may be possible to identify a region modulating the effect of salicylate and, potentially, essential amino acid residues that dictate salicylate binding. For example,

should a topo IIα mutant containing the C-terminal domain of topo IIβ be insensitive to salicylate inhibition, then it is likely that the binding site of salicylate is located within or is influenced by sequences within the C-terminal domain. Previous studies have also used similar methods to isolate topo II mutants resistant to doxorubicin and mAMSA (Gilroy et al., 2006; Patel et al., 1997). It is also interesting that these mutations (for example, G465D in topo IIβ) can also have phenotypic consequences, such as decreased ATP hydrolysis (Gilroy et al., 2006). Therefore, identification of similar sites necessary for topo II inhibition by salicylate may also lead to new insights into topo II biochemical function.

6.4.2 Identifying novel chemical relationships required for topoisomerase II inhibition

In this thesis, chemical modifications that influence the *in cyto* and *in vitro* inhibition of topo II by salicylate-based compounds were examined. While the evaluation was limited by the commercial availability of these compounds, the findings point towards key structural features. Further work would require more systematic medical chemistry approaches, but identifying these chemical properties could aid the development of novel topo II catalytic inhibitors.

In addition to serendipitous identification, topo II-targeting agents have also been discovered using high-throughput screening of compound libraries, a task that is both labour and resource intensive. An alternative approach is to design novel topo II-targeting agents through strategic chemical modifications structurally-based on established inhibitors. An example of this approach is highlighted by the development of QAP1, an ATP-binding competitor with high affinity for the topo II-ATP binding pocket (Chène et al., 2009; Furet et al., 2009). Using computer-guided modeling of the nucleotide-binding pocket, researchers chemically modified aminopurine analogs, which were then

individually tested for their ability to inhibit topo II, ultimately leading to the identification of QAP1.

In light of the data in this thesis examining the salicylate-related compounds, an expanded and directed effort could be utilized to develop novel topo II catalytic inhibitors. For example, the effect of an extended carbon bridge between the aromatic ring and hydroxyl group at the 2'-position has not yet been examined. Alternatively, using anthranilic acid (*o*-amino-benzoic acid), the effect of substituting an amino group at the 2'-position could be examined, particularly as the amino group can also participate in hydrogen bonding. In our work, we also examined two clinically used, anthranilic acid derived NSAIDs, tolfenamic acid and flufenamic acid and whether these compounds could attenuate doxorubicin-induced DNA damage signaling (Appendix 1). Neither of these compounds had effects on attenuating this DNA damage signaling, however, both compounds contain large, bulky substituents, blocking the 2'-position. This blockage appears to support our earlier findings that the 2'-position must contain a free hydrogen bond donating substituent to be an effective topo II catalytic inhibitor, which is absent in both instances with these two NSAIDs.

There appears to be a subtle relationship between the placement of hydroxyl groups on the benzoic acid ring and the ability to act as either a topo II catalytic inhibitor or a topo II poison. While 2,3,4-THBA acts as a catalytic inhibitor, 3,4,5-THBA is a topo II poison (Bandele and Osheroff, 2008; López-Lázaro et al., 2011). It is particularly interesting that the loss of the 2'-moiety and gain of a 5'-substituent shifts the compound from behaving as a catalytic inhibitor to a poison. By definition, poisons stabilize a topo II covalent cleavable complex with DNA, while inhibitors do not. Thus, it is curious that such a subtle modification is associated with such a significant change in biochemical behaviour. One

possibility is that the salicylate-based compounds examined in this thesis may bind to a site on topo II different from that to which 3,4,5-THBA binds. The specific orientation of the hydroxyl groups may indeed enhance the affinity for one binding site over another. A second possibility is that both compounds bind within the same site, yet the orientation of the hydroxyl groups provide for different non-covalent amino acid interactions to be made in the context of topo II. As certain amino acid residues have been found to be critical for mediating the topo II cleavage transition state, it is possible that interference with the transition state may lead to poisoning over catalytic inhibition. For example, if the nucleophilic attack by the catalytic tyrosine residue on the phosphate backbone is impeded, then the DNA break is never created. In contrast, if the DNA break occurs but then is trapped (such as in the case of etoposide), the compound acts as a topo II poison. This hypothesis becomes more challenging to test without more technologically advanced docking and binding studies. It remains unknown whether 3,4,5-THBA is an isoform-specific topo II poison. If 3,4,5-THBA were to be isoform-specific, further study of this and related compounds might provide insight into the development of novel topo IIα-specific poisons.

Other modifications could be explored that may shift the behaviour of a drug to favour its inhibition of topo II over its other known mechanisms of action. Sulfasalazine, for example, is an anti-inflammatory agent that contains an azo-bond, which is hydrolyzed *in vivo* to form two metabolites: sulfapyridine and 5-ASA. Of these, 5-ASA is believed to confer the anti-inflammatory properties (Rachmilewitz, 1989; Schroeder et al., 1987). However, the results shown here suggest that these metabolites do not inhibit topo II. For sulfasalazine, if one were to chemically convert the azo-bond to a non-hydrolyzable

alternative, such as a carbon double bond, this variant might allow for sustained inhibition of topo II *in vivo*.

Whether the salicylate-related compounds tested in this thesis also exhibit specificity for the alpha isoform remains unknown. A thorough and expanded screen of these compounds may identify a broader class of topo IIα-specific inhibitors, which, in conjunction with the future studies outlined earlier, will provide a better understanding of whether specific chemical substituents determine whether a compound selectively targets the topo IIα or topo IIβ isoform.

6.4.3 Clinical implications of salicylates as catalytic inhibitors of topoisomerase II

A broader implication of this work is whether salicylates can be used clinically as topo II catalytic inhibitors, or whether this property poses a concern for their potential impact on the efficacy of topo II poison-based chemotherapy regimens. It is clear from the current body of literature that selected topo II catalytic inhibitors can be beneficial for reducing some of the negative side effects associated with topo II poisons. Studies have demonstrated that dexrazoxane can decrease doxorubicin-mediated cardiotoxicity (Lipshultz et al., 2004, 2010), myelosuppression (Hofland et al., 2005a), and extravasation (Langer et al., 2012) without compromising chemotherapeutic efficacy. However, as demonstrated by this thesis work and others, pretreatment of cells with other catalytic inhibitors, followed by concurrent treatment with topo II poisons, can lead to decreased cytotoxicity (Bau and Kurz, 2011; Vavrova et al., 2013). Indeed, this effect is observed *in cyto* with salicylate. Whether salicylate co-administration negatively impacts the effectiveness of topo II poisons *in vivo* remains to be seen. To elucidate this, studies in murine models could be conducted using human tumor xenografts and comparing animals

treated with a topo II poison alone with those receiving salicylate before and during treatment with a topo II poison. The effects of daily administration of salicylates on tumor size, progression and chemotherapeutic efficacy could be determined.

Our data with salicylate-related compounds, in particular sulfasalazine and diflunisal, suggest that our findings with salicylate may be extended to a broader class of compounds. Many of the salicylates and related hydroxybenzoic acids studied in this work are found in plant-based foods, from vegetables and spices to teas. Of concern is the potential impact the ingestion of salicylates from pharmaceutical or dietary sources might have if taken during treatment with topo II poison-based chemotherapeutics.

Until studies investigating the impact of salicylates on chemotherapeutic efficacy have been completed, the beneficial effects of salicylates (such as aspirin) cannot be overlooked. The anti-inflammatory properties of these compounds are clinically effective in the management of many inflammatory conditions, while daily low-dose aspirin supplementation is widely used to decrease adverse cardiovascular and cerebrovascular events. In addition, there is now an increasing body of literature that suggests that regular aspirin supplementation decreases cancer incidence and may even prolong disease-free survival after cancer diagnosis (Birmann et al., 2014; Holmes et al., 2010; Rothwell et al., 2010, 2011).

6.5 The future of topoisomerase II catalytic inhibitors as part of an anti-cancer strategy

Topo II catalytic inhibitors were first identified almost thirty years ago, with considerable anticipation for their clinical utility. Unfortunately, the few catalytic inhibitors that entered clinical trials were subsequently abandoned primarily due to poor

outcomes. The exception was the identification of dexrazoxane and its clinical utility in decreasing the dose-limiting cardiotoxicity associated with anthracycline therapy. Despite this success with dexrazoxane, a recent study suggests that only 2% of pediatric patients are prescribed dexrazoxane as an adjuvant to doxorubicin treatment (Lipshultz et al., 2013) and studies in adult populations are lacking. Other catalytic inhibitors, such as merbarone, were tested in clinical trials as single agent therapy, largely based on preclinical studies demonstrating their cytotoxicity when administered alone (Chang et al., 1993; Jones et al., 1993; Kraut et al., 1992; Malik et al., 1997). More recent studies with vescalagin, α-heterocyclic thiosemicarbazones and N-fused imidazoles have also measured single-agent cell cytotoxicity as determinants for whether these compounds hold chemotherapeutic potential (Auzanneau et al., 2012; Baviskar et al., 2011; Huang et al., 2010). Aside from the studies with vescalagin (Auzanneau et al., 2012), other studies identifying novel topo II catalytic inhibitors typically do not examine isoform specificity. On the basis of our current understanding of topo II catalytic inhibitors and their ability to protect cells from topo II poisons, it is curious to note whether these compounds should be studied as chemoprotective agents instead of applying them as anti-tumor chemotherapeutics. Catalytic inhibitors may be of better use when co-administered with poisons to exploit potential cardioprotective properties or to counter extravasation injuries that are common with topo II poison treatment. As such, future studies might benefit from redesigning study protocols to examine the concurrent use of both catalytic inhibitors and poisons. This is especially necessary, as it has been found that in the general adult population, long-term cardiac-related side effects are associated with up to 10% of patients after therapy with topo II poisons (Yeh, 2006).

Another avenue of study is to revisit previously characterized catalytic inhibitors to determine whether these compounds have isoform selectivity. Previously documented catalytic inhibitors have primarily been studied in the context of topo IIα and have not been evaluated for their effects on topo IIβ. Studies have demonstrated that dexrazoxane selectively degrades topo IIβ while leaving topo IIα unaffected, despite effectively inhibiting either isoform *in vitro* (Lyu et al., 2007). Thus, the bisdioxopiperazines display a higher-order level of enzyme control, independent from direct enzyme inhibition; it may be that other catalytic inhibitors have similar previously uncharacterized properties. Expanded characterization of these drugs may ultimately increase the versatility of this class of compounds, both from a scientific and clinical perspective. Catalytic inhibitors targeting the topo IIα isoform may aid experiments aimed at better understanding topo IIα-dependent processes during replication. As topo IIα is notoriously difficult to manipulate genetically, pharmacological inhibition of this isoform may be a useful tool for selectively studying topo IIα in cell models. Similarly, topo IIβ-selective catalytic inhibitors may allow for the nuances of the role of topo IIβ in regulated transcription and neural development to be investigated and may prove to have considerable impact on mitigating the significant long-term side effects associated with chemotherapy regimens involving topo II poisons. A therapeutic strategy could be envisioned where individuals are treated with a topo IIβ-selective catalytic inhibitor prior to treatment with a topo II poison. This would prevent the formation of topo IIβ-DNA cleavable complexes, while topo IIα-mediated strand breaks would still be induced, achieving a better clinical outcome.

6.6 Final thoughts and conclusions

The work presented in this thesis significantly enhances our understanding of the salicylates through identification of a novel target of inhibition. Through this work, I have described the biochemical mechanism by which salicylate functions as a novel catalytic inhibitor of topo IIα. Given the importance of topo II in cell proliferation and the widespread prevalence of salicylates, this work provides a basis on which future studies can build to examine the impact of topo II catalytic inhibitors on clinical chemotherapy.

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Appendix 1: Examination of the effects of non-steroidal anti-inflammatory drugs on doxorubicin-induced DNA damage signaling

Although early work in this thesis supported the conclusion that the effects of salicylate on the catalytic activity of topo II were not attributable to its actions as an anti-inflammatory (Chapter 3, Figure 3.3), the widespread availability and use of non-steroidal anti-inflammatory drugs (NSAIDs) and the observed impact of salicylate on doxorubicin efficacy *in cyto* warranted examination of these agents.

NSAIDs can be salicylate-based (such as those examined in Chapter 5) or non-salicylate-based. The latter are further sub-classified based on their chemical structures. These classifications include: propionic acid (*e.g.* ibuprofen), acetic acid (*e.g.* indomethacin), anthranilic acid (*e.g.* flufenamic and tolfenamic acids) and enolic acid (*e.g.* piroxicam) (Fig. A1). NSAIDs also include the COX-2 selective inhibitors, including celecoxib and rofecoxib; however, as these were examined in Chapter 3 they are not included here. The effect of acetaminophen, an analgesic and anti-pyretic of widespread use but that does not have anti-inflammatory activity, was also examined.

The effects of these non-salicylate-based NSAIDs on doxorubicin-induced DNA damage signaling were examined. In studies similar to those described in Chapters 3 and 5, MCF-7 cells were pretreated with increasing concentrations of NSAIDs for one hour prior to the addition of doxorubicin (1 μM) and continued incubation for an additional two hours. Whole cell lysates were collected and immunoblotted for markers of the DNA damage response (ATM, Chk2, SMC1 and p53). Pretreatment of cells with acetaminophen, indomethacin, piroxicam or ibuprofen had no effect on doxorubicin-induced DNA damage

signaling (Fig. A2). Similarly, these compounds do not inhibit topo II decatenation activity *in vitro* (E. Kurz, unpublished observation).

Flufenamic acid and tolfenamic acid are classified as anthranilic acids; however, they also resemble 2'-modified salicylate molecules (Fig. A1). Salsalate is a salicylate-based NSAID that consists of two salicylate molecules, one linked via the 2'-moiety, the other to the carboxylic acid (Fig. A1). Pretreatment of cells with salsalate, flufenamic acid or tolfenamic acid decreased doxorubicin-induced p53 phosphorylation but not phosphorylation of Chk2 (Fig. A3). None of the other DNA damage substrates examined were affected by pretreatment with these three compounds. All three of these compounds contain bulky substituents at the 2'-position. As we noted in the discussion for Chapter 5, the presence of an electronegative group capable of hydrogen bonding (such as a hydroxyl group) at this position is important for topo II inhibition and, as a follow-on effect, decreased doxorubicin-induced DNA damage signaling. While salsalate is efficiently hydrolyzed into two salicylate molecules in patients (Dromgoole et al., 1984), whether this occurs in cultured cells is unknown. It is speculated that the ability of these salicylate-like compounds to act as catalytic inhibitors of topo II is likely impaired due to their blocked 2'-position.

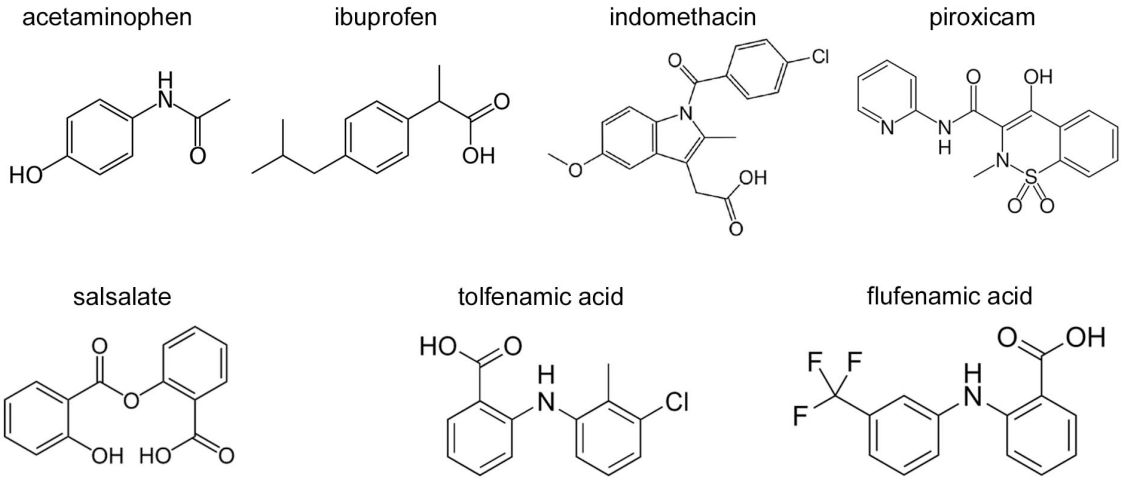

Figure A1. Structures of non-steroidal anti-inflammatory drugs examined.

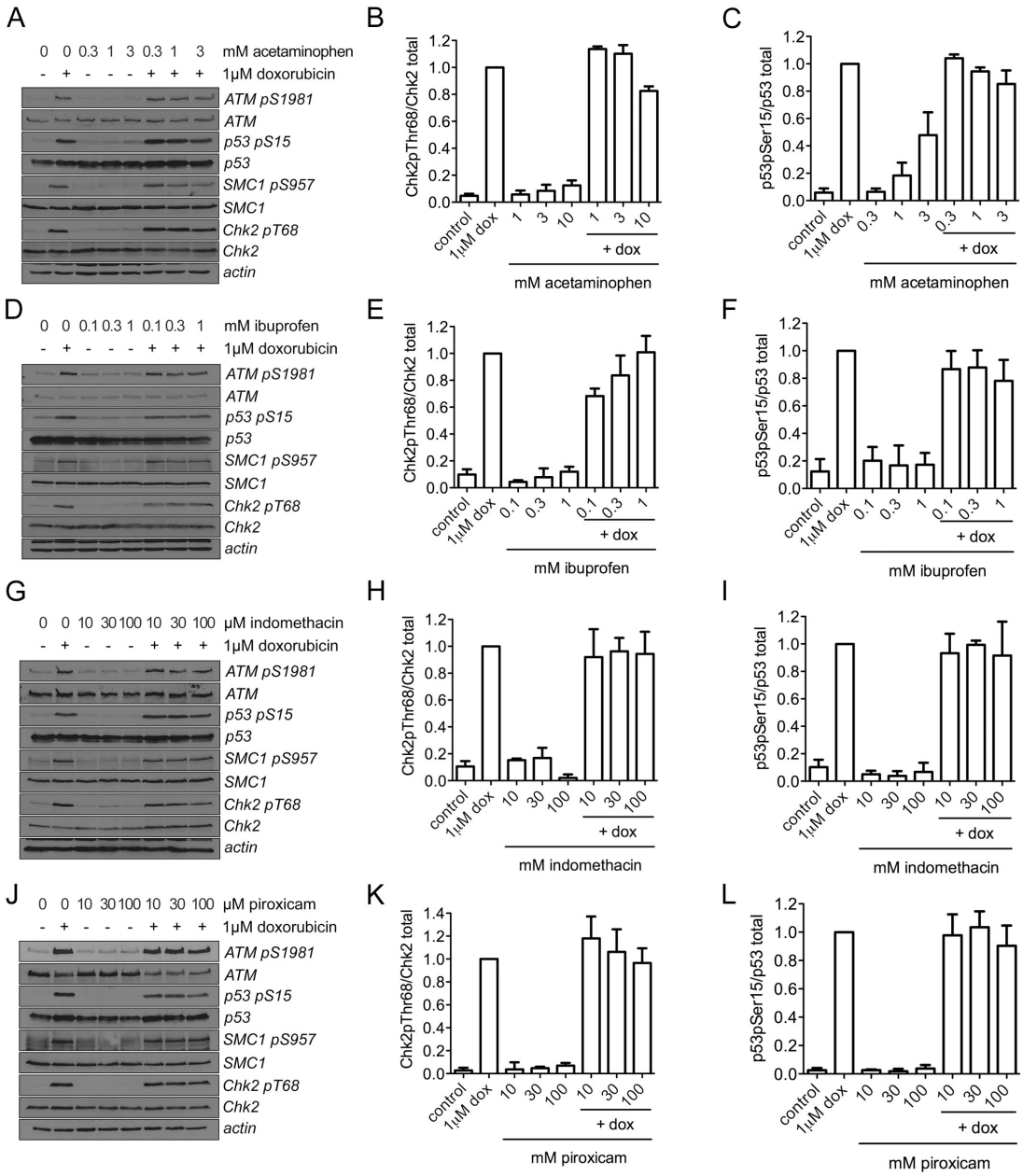
A
0 0 0.3 1 3 0.3 1 3 mM acetaminophen
- + - - - + + + 1μM doxorubicin
ATM pS1981
ATM
p53 pS15
p53
SMC1 pS957
SMC1
Chk2 pT68
Chk2
actin
B
Chk2pThr68/Chk2 total
control
1μM dox
1 3 10 1 3 10
+ dox
mM acetaminophen
C
p53pSer15/p53 total
control
1μM dox
0.3 1 3 0.3 1 3
+ dox
mM acetaminophen
D
0 0 0.1 0.3 1 0.1 0.3 1 mM ibuprofen
- + - - - + + + 1μM doxorubicin
ATM pS1981
ATM
p53 pS15
p53
SMC1 pS957
SMC1
Chk2 pT68
Chk2
actin
E
Chk2pThr68/Chk2 total
control
1μM dox
0.1 0.3 1 0.1 0.3 1
+ dox
mM ibuprofen
F
p53pSer15/p53 total
control
1μM dox
0.1 0.3 1 0.1 0.3 1
+ dox
mM ibuprofen
G
0 0 10 30 100 10 30 100 μM indomethacin
- + - - - + + + 1μM doxorubicin
ATM pS1981
ATM
p53 pS15
p53
SMC1 pS957
SMC1
Chk2 pT68
Chk2
actin
H
Chk2pThr68/Chk2 total
control
1μM dox
10 30 100 10 30 100
+ dox
mM indomethacin
I
p53pSer15/p53 total
control
1μM dox
10 30 100 10 30 100
+ dox
mM indomethacin
J
0 0 10 30 100 10 30 100 μM piroxicam
- + - - - + + + 1μM doxorubicin
ATM pS1981
ATM
p53 pS15
p53
SMC1 pS957
SMC1
Chk2 pT68
Chk2
actin
K
Chk2pThr68/Chk2 total
control
1μM dox
10 30 100 10 30 100
+ dox
mM piroxicam
L
p53pSer15/p53 total
control
1μM dox
10 30 100 10 30 100
+ dox
mM piroxicam

Figure A2. Acetaminophen, ibuprofen, indomethacin and piroxicam do not attenuate doxorubicin-induced DNA damage signaling.

Human breast cancer (MCF-7) cells were treated with increasing concentrations of acetaminophen (A), ibuprofen (D), indomethacin (G) or piroxicam (J) for 1 h prior to the addition of doxorubicin (1μM) and continued incubation for an additional 2 h. Whole cell lysates were collected and immunoblotted with phosphospecific and pan-specific antisera to ATM (phosphorylated on serine 1981), p53 (phosphorylated on serine 15), SMC1 (phosphorylated on serine 957) and Chk2 (phosphorylated on threonine 68). Immunoblotting for actin was used as a loading control. A representative experiment is shown for each compound. The immunoblots from at least three independent replicates for Chk2 and p53 were scanned, analysed densitometrically and plotted (acetaminophen (B and C); ibuprofen (E and F); indomethacin (H and I); piroxicam (K and L)). Data are shown as mean values and the error bars represent the S.E.M. No statistical significance of pretreatment effects compared with doxorubicin alone was observed as determined by one-way ANOVA with a Tukey post-hoc test.

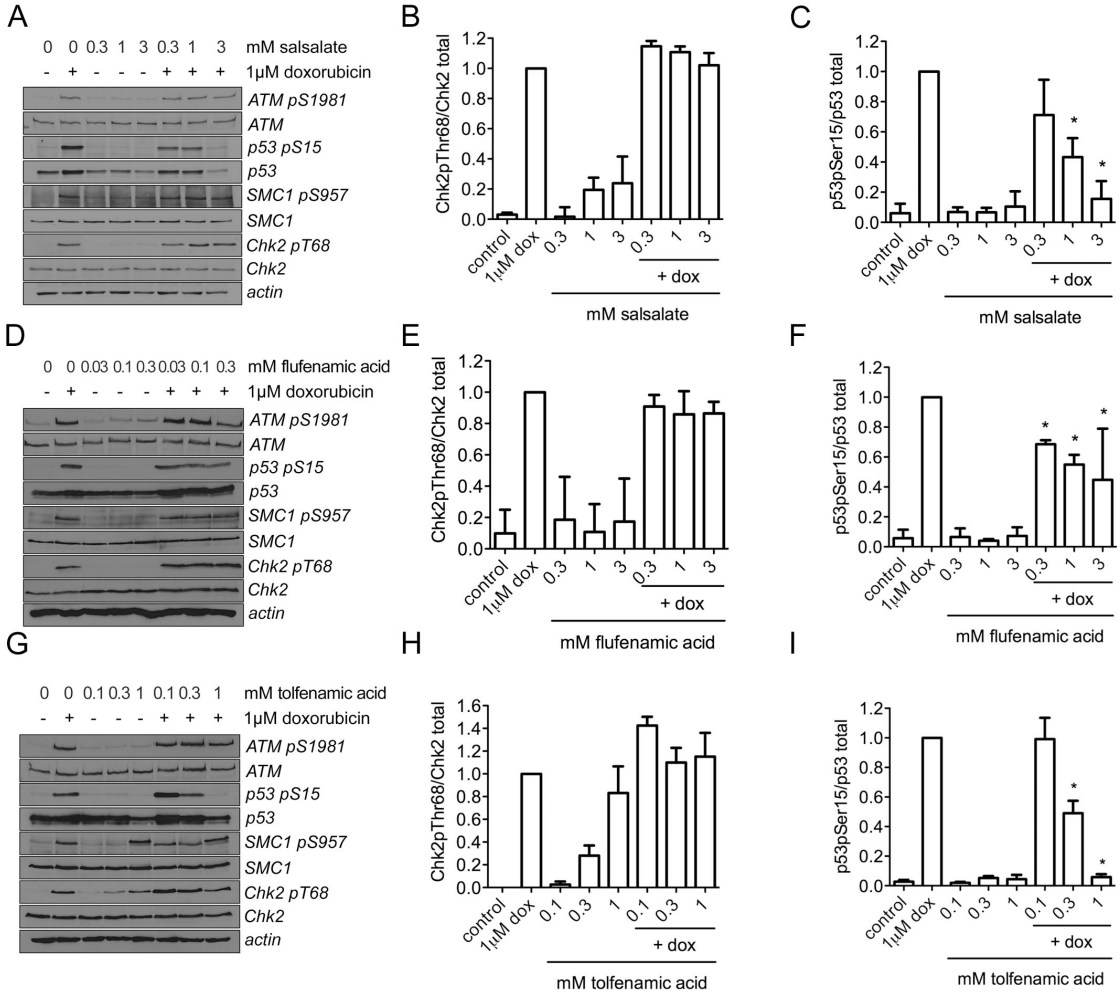
A
0 0 0.3 1 3 0.3 1 3 mM salsalate
- + - - - + + + 1μM doxorubicin
ATM pS1981
ATM
p53 pS15
p53
SMC1 pS957
SMC1
Chk2 pT68
Chk2
actin
B
Chk2pThr68/Chk2 total
control
1μM dox
0.3
1
3
0.3
1
3
+ dox
mM salsalate
C
p53pSer15/p53 total
control
1μM dox
0.3
1
3
0.3
1
3
+ dox
mM salsalate
D
0 0 0.03 0.1 0.3 0.03 0.1 0.3 mM flufenamic acid
- + - - - + + + 1μM doxorubicin
ATM pS1981
ATM
p53 pS15
p53
SMC1 pS957
SMC1
Chk2 pT68
Chk2
actin
E
Chk2pThr68/Chk2 total
control
1μM dox
0.3
1
3
0.3
1
3
+ dox
mM flufenamic acid
F
p53pSer15/p53 total
control
1μM dox
0.3
1
3
0.3
1
3
+ dox
mM flufenamic acid
G
0 0 0.1 0.3 1 0.1 0.3 1 mM tolfenamic acid
- + - - - + + + 1μM doxorubicin
ATM pS1981
ATM
p53 pS15
p53
SMC1 pS957
SMC1
Chk2 pT68
Chk2
actin
H
Chk2pThr68/Chk2 total
control
1μM dox
0.1
0.3
1
0.1
0.3
1
+ dox
mM tolfenamic acid
I
p53pSer15/p53 total
control
1μM dox
0.1
0.3
1
0.1
0.3
1
+ dox
mM tolfenamic acid

Figure A3. Salsalate, flufenamic acid and tolfenamic acid do not attenuate doxorubicin-induced DNA damage signaling.

Human breast cancer (MCF-7) cells were treated with increasing concentrations of salsalate (A), flufenamic acid (D), or tolfenamic acid (G) for 1 h prior to the addition of doxorubicin (1μM) and continued incubation for an additional 2 h. Whole cell lysates were collected and immunoblotted with phosphospecific and pan-specific antisera to ATM (phosphorylated on serine 1981), p53 (phosphorylated on serine 15), SMC1 (phosphorylated on serine 957) and Chk2 (phosphorylated on threonine 68). Immunoblotting for actin was used as a loading control. A representative experiment is shown for each compound. The immunoblots from at least three independent replicates for Chk2 and p53 were scanned, analysed densitometrically and plotted (salsalate (B and C); flufenamic acid (E and F); tolfenamic acid (H and I)). Data are shown as mean values and the error bars represent the S.E.M. Statistical significance of pretreatment effects compared with doxorubicin alone was determined by one-way ANOVA with a Tukey post-hoc test and is denoted by the asterisk (*; $p < 0.05$).