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Sodium salicylate is a novel catalytic inhibitor of human DNA topoisomerase II alpha

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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 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 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.

1. 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; EC 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 [1–3]. 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 [2,4]. As a result of this, these drugs

Abbreviations: 5-IDNR, 5-iminodaunorubicin; ASA, acetylsalicylic acid; ATM, ataxia telangiectasia mutated; COX-2, cyclooxygenase-2; CPT, camptothecin; DEM, diethyl maleate; DSB, double-stranded break; ICE, in vivo complex of enzyme; IR, ionizing radiation; kDNA, kinetoplast DNA ;; MTX, mitoxantrone; NAC, N-acetyl cysteine; NFκB, nuclear factor kappa B; NSAID, non-steroidal anti-inflammatory drug; PIKKs, phosphoinositide 3-kinase-like kinases; SS, sodium salicylate; TNFα, tumor necrosis factor alpha; topo II, topoisomerase IIα; TPT, topotecan; VP-16, etoposide.

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'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 [4,5]. 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 [6]. 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 [7].

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 (COX-2) and nuclear factor kappa B (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.

2. Materials and methods

2.1. 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).

2.2. 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, ON, Canada).

2.3. 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).

2.4. Protein extraction and immunoblotting

Whole cell extracts were prepared from logarithmically growing MCF-7 cells as previously described [6]. Electrophoresis and immunoblotting conditions were as previously described [6].

2.5. Image analysis

Image analysis was performed using ImageQuant software (GE Healthcare Life Sciences, Baie d'Urfe, QC, 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.

2.6. 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 manu-

facturer's protocol with the following modifications: post-treatment, cells were trypsinized, washed once with PBS and counted; 5×10^3 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 20 V (1 V/cm) and at 4 °C. Comets were visualized using a Leica Microsystems DMRXA2 fluorescence microscope (Richmond Hill, ON, Canada). Measurement of tail moments was done using ImageJ [8] or CometScore™ (www.autocomet.com; TriTek Corporation, Sumerduck, VA, USA). A minimum of 50 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.

2.7. In vivo complex of enzyme bioassay

Formation of stabilized topo II cleavable complexes was examined using the in vivo complex of enzyme (ICE) bioassay [9]. 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 × *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.

2.8. Topo II-mediated DNA cleavage assay

The effects of sodium salicylate on topo II catalytic activity were analyzed based on a protocol modified from [10] as previously described [11,12]. 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 10 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 8 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 [11,12]. 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.

2.9. Topo 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 comparison 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 $MgCl_2$, 0.5 mM 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 10 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 15 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 100 V for 40 min and visualized with ethidium bromide.

2.10. 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 pretreatment 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. Results

3.1. 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 [6], 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 [6], treatment of MCF-7 cells with doxorubicin led to the autophosphorylation of ATM (on ser1981) 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. 1A and data not shown). As observed with the human lymphoblastoid cells [6], 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 abolished doxorubicin-induced phosphorylation of this kinase and its downstream effectors (Fig. 1B).

To investigate the effects of four known hydroxyl radical scavengers on this drug-induced signalling [13–15], 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. 1C) or sodium salicylate (Fig. 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. 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. 1E).

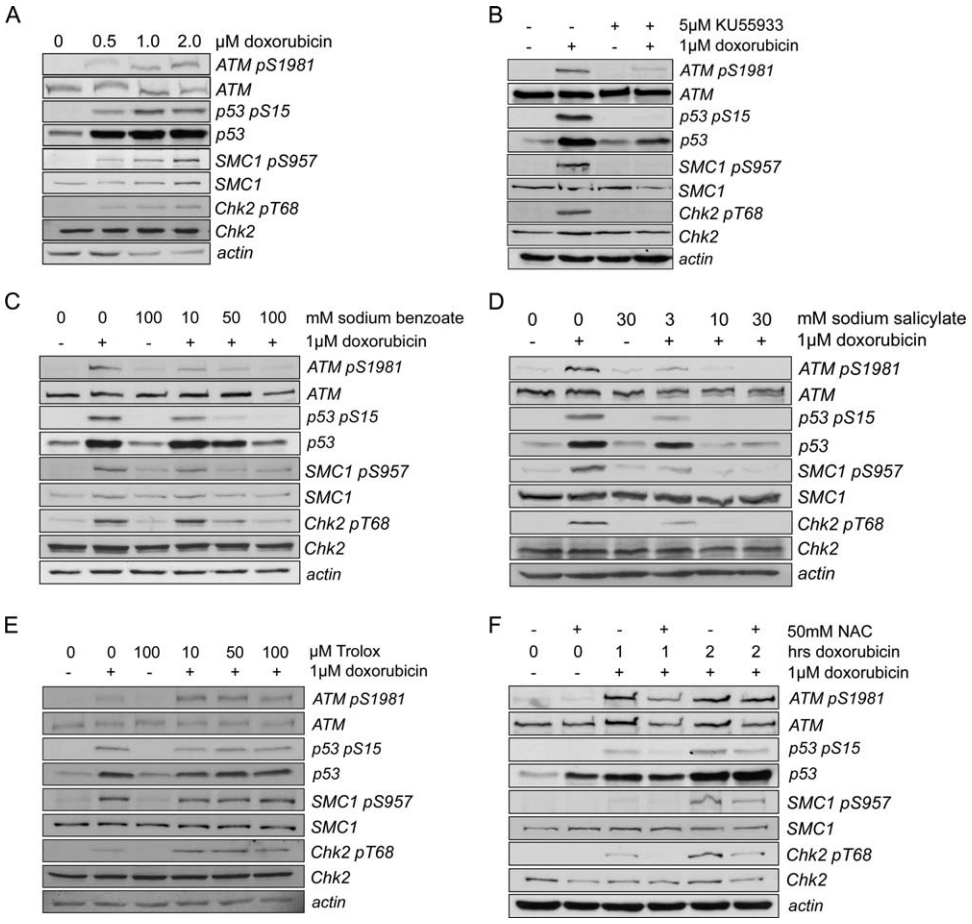

Fig. 1. Doxorubicin-induced DNA damage signalling in MCF-7 cells is dependent on the ATM protein kinase and is attenuated by pretreatment of cells with NAC, sodium benzoate or sodium salicylate, but not Trolox™. (A) Human MCF-7 breast cancer cells were treated with increasing concentrations of doxorubicin 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 (+, 1 μM) and further incubation for 1 or 2 h. Whole cell extracts were prepared and analyzed by immunoblotting as described in (A).

3.2. 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 [5]. 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 [16,17]. 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. 2A and 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).

3.3. Sodium salicylate attenuates DNA damage signalling from multiple classes of topo 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. 2B and S1B). In a manner similar to that observed for doxorubicin, pretreatment of cells with sodium salicylate attenuated the phosphorylation of all downstream effectors of ATM, with the 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,

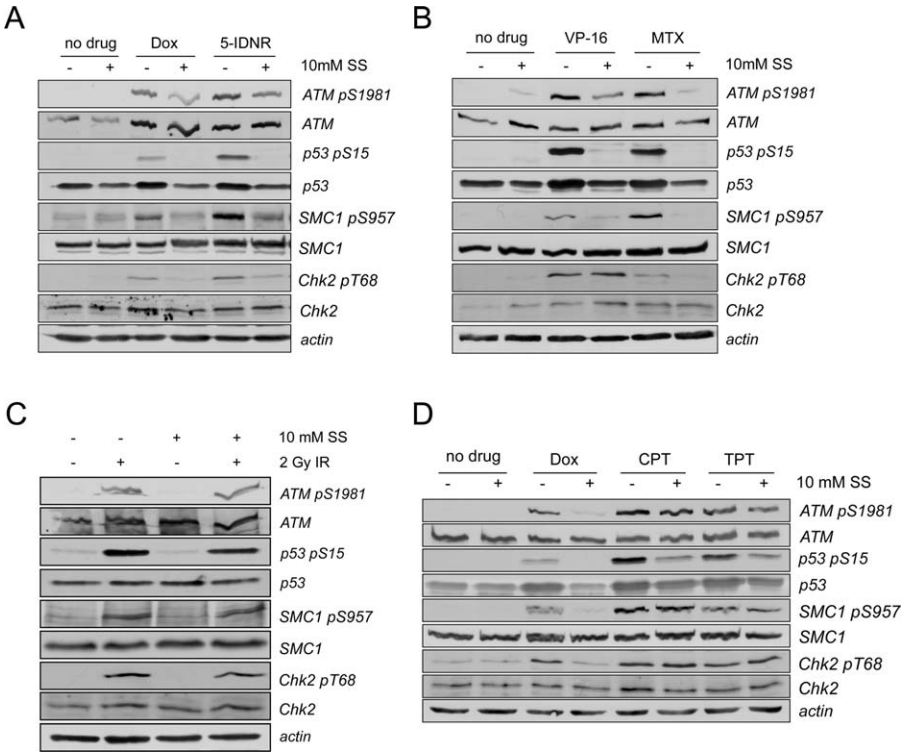

Fig. 2. Attenuation of DNA damage signalling by sodium salicylate is independent of reactive oxygen species and is observed with multiple topo II poisons, but not IR or topoisomerase I poisons. (A) MCF-7 cells were pretreated (+) or not (−) with sodium salicylate (SS, 10 mM) for 1 h prior to the addition 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. 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. 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. 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. 1.

but is more generally observed across multiple classes of topo II poisons.

3.4. Inhibition of DNA damage signalling by sodium salicylate is specific for topo 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 [18,19]. 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 h) prior to exposure to ionizing radiation (IR) (2 Gy, 15 min recovery) (Figs. 2C and S1C). 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. 2C and 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 an effect on topoisomerases in general, MCF-7 cells were pretreated with sodium salicylate (10 mM, 1 h) prior to exposure to doxorubicin (1 μM, 2 h) or the topoisomerase I poisons, camptothecin (10 μM, 2 h) or topotecan (0.2 μM, 2 h) (Figs. 2D and 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. 2D and S1D).

3.5. 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 COX-2 [20], as well as inhibiting the activation of NFκB through inhibition of IKKβ kinase, which normally phosphorylates the NFκB inhibitor IκBα to release active NFκB [21,22]. 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. 3A). In contrast, pretreatment of cells with diethyl maleate, another characterized inhibitor of NFκB [23,24], led to more robust (40%) inhibition of TNFα-induced phosphorylation of IκBα (Fig. 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 (50 μM), a COX-2 inhibitor [25], or diethyl maleate (0.5 mM) for 1 h prior to the addition of doxorubicin (1 μM, 2 h) (Fig. 3B and C). In contrast to sodium salicylate, neither rofecoxib nor diethyl maleate pretreatment of cells affected doxorubicin-induced DNA damage signalling, suggesting that the effects observed with sodium salicylate are independent of its capacity to inhibit COX-2 and NFκB.

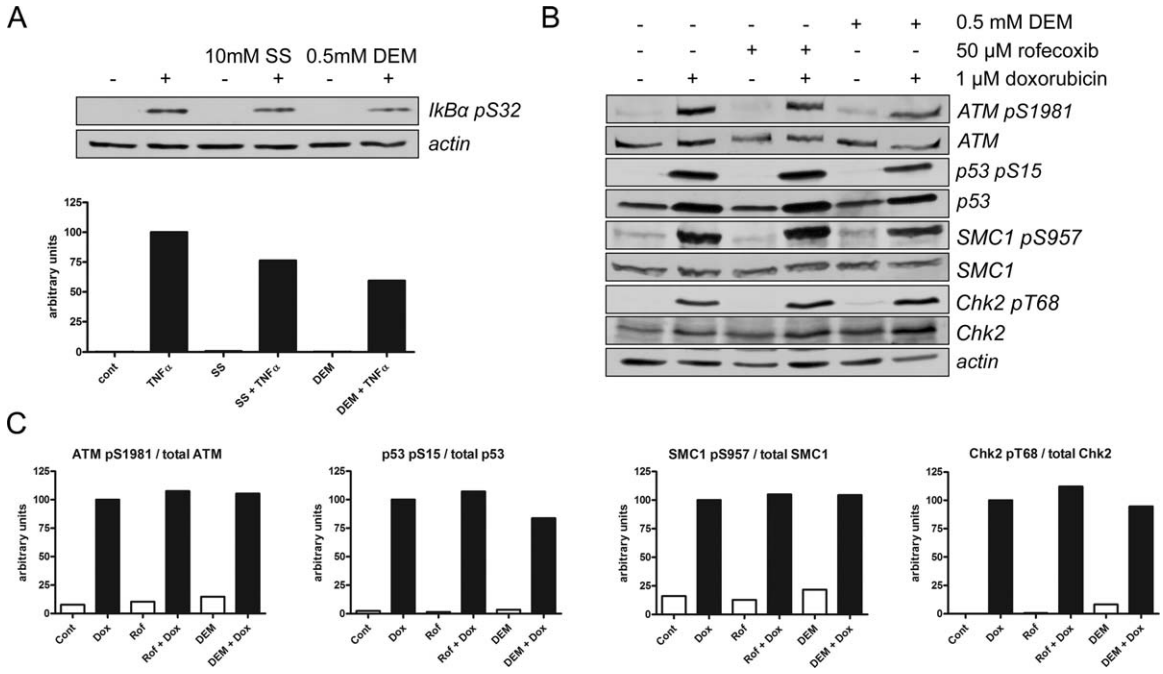

Fig. 3. Sodium salicylate attenuation of DNA damage signalling is not mediated by inhibition of cyclooxygenase-2 or NFκB. (A) MCF-7 cells were pretreated with sodium salicylate (SS, 10 mM) or diethyl maleate (DEM, 0.5 mM) for 1 h prior to stimulation with TNFα (20 ng/mL) and continued incubation for 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. 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.

3.6. 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. 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. 4). However, when MCF-7 cells were incubated with sodium salicylate prior to doxorubicin treatment, DNA DSB formation was completely abrogated (Fig. 4). Together, these data demonstrate that pretreatment of cells with sodium salicylate prevents the induction of DNA DSBs by doxorubicin.

3.7. 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 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

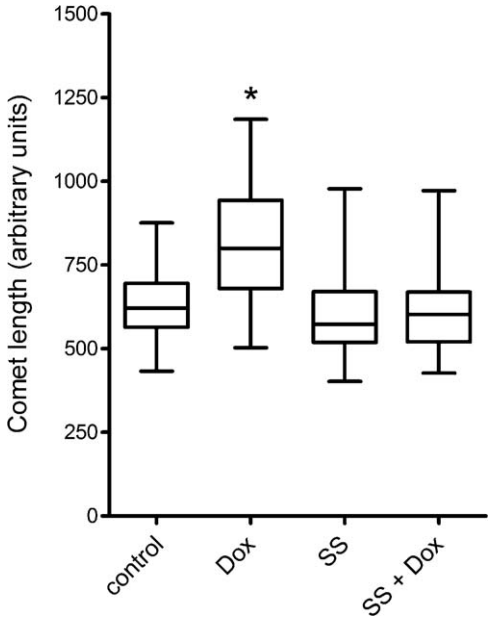

Fig. 4. Sodium salicylate prevents the formation of doxorubicin-induced DNA DSBs. MCF-7 cells were pretreated or not with sodium salicylate (SS, 10 mM) for 1 h prior to the addition of doxorubicin (Dox, 1 μM) or vehicle control (control; DMSO) and continued incubation for 2 h. Cells were then washed, 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 ($^*p < 0.0001$).

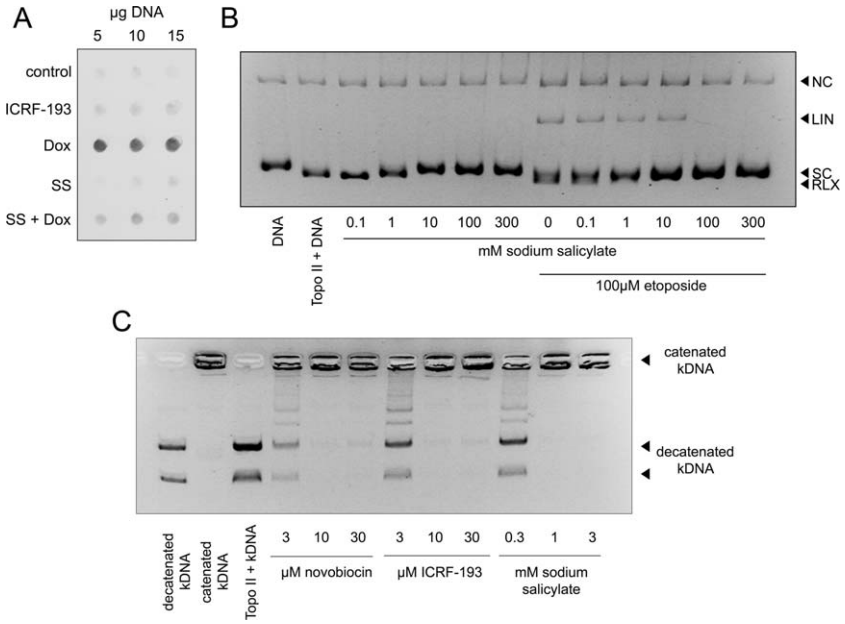

Fig. 5. Sodium salicylate attenuates doxorubicin-stabilized topo II–DNA cleavable complex formation and inhibits topo II catalytic activity. (A) MCF-7 cells were pretreated or not with sodium salicylate (SS, 10 mM, 1 h) prior to the addition of doxorubicin (1 μM) and continued incubation for an additional 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. (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), linear (LIN) and nicked circular (NC) DNA species. (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.

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. 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. 5A), suggesting that sodium salicylate abrogates doxorubicin-stabilization of cleavable complexes, thereby preventing the formation of DSBs.

3.8. Sodium salicylate inhibits topo 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 [10–12]. 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 relaxation (Fig. 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. 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. 5B). In a manner similar to previously characterized topo II catalytic inhibitors [26], 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. 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. 5C).

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

Given our demonstration that sodium salicylate inhibits DNA DSB induction by doxorubicin (Fig. 4) and decreases in vivo cleavable complex formation (Fig. 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. 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. 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. 6B). This effect was not limited to

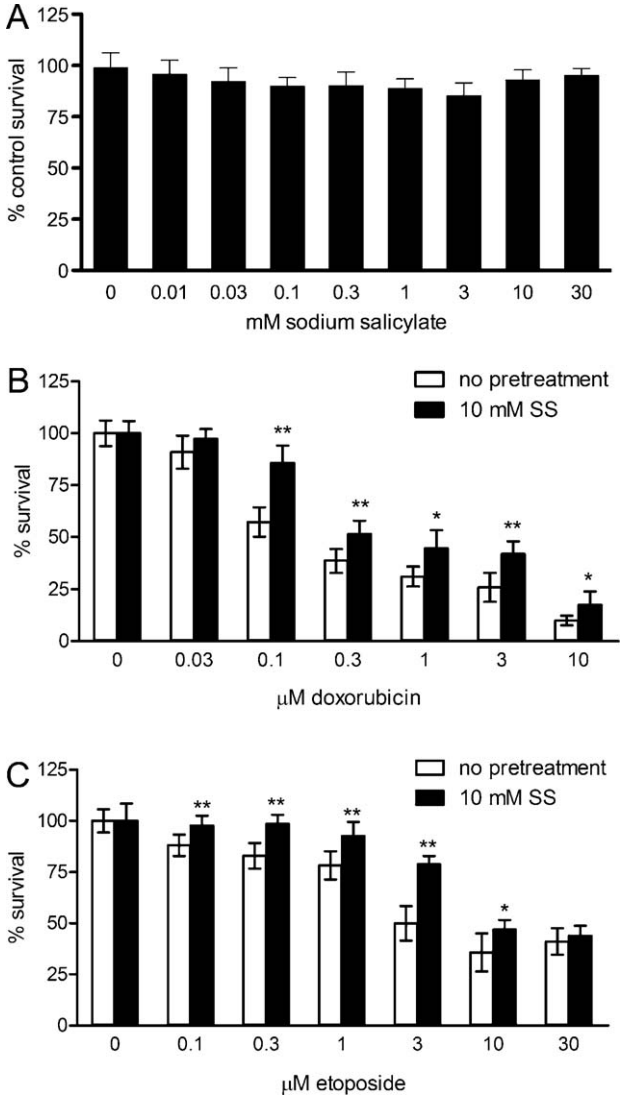

Fig. 6. Sodium salicylate attenuates doxorubicin- and etoposide-induced cytotoxicity. MCF-7 cells were seeded in 96-well plates at 5000 cells per well 16 h prior to drug treatment. (A) In replicates of eight, cells were treated with increasing concentrations of sodium salicylate for 3 h. Following treatment, 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 ($^{*}p < 0.01$; $^{**}p < 0.001$). Each experiment was performed three times. Representative experiments are shown.

doxorubicin, as pretreatment of cells with sodium salicylate also conferred protection from etoposide cytotoxicity (Fig. 6C).

4. 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 [3]. 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.

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 [6]. 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 [6]. 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 [13–15].

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 [14]. It has also been shown to reduce the cytotoxicity of teniposide, an epipodophyllotoxin-related topo II poison [27]. 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) [27]. 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 [28,29]. 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 the 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 [30]. 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 [31].

Compounds that target topo II are classified into two categories: topo II poisons and topo II inhibitors (reviewed in [4,32]). 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 [33].

Sodium salicylate has also been extensively characterized as an inhibitor of NFκB activation [21,22]. Activation of NFκB is accompanied by the release from its inhibitory partner IκBα following IκBα phosphorylation [34]; 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 [23,24], 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 [35] and for short duration (3–7 days) treatments [36,37], and can reach low millimolar concentrations in xenograft tumor sites in athymic mice following oral administration of a single low dose of ASA [38]. 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.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2010.10.009.

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