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

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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.

1. 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 [1,2]. 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 [1,2]. Topo IIα has key roles in cell division and DNA replication, while topo IIβ is important in regulated transcription and neuronal development [3,4].

Targeting of topo II has proven to be an effective approach in cancer chemotherapy [5,6]. 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 [7,8]. 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 [7]. While topo II poisons have been long-time mainstays of many chemotherapy regimens, catalytic

Abbreviations: 2-FBA, 2-fluorobenzoate; 2-MBA, 2-methylbenzoate; 5-ASA, 5-aminosalicylic acid; ASA, acetylsalicylic acid; DHBA, dihydroxybenzoate; HBA, hydroxybenzoate; ICE, in vivo complex of enzyme; kDNA, kinetoplast DNA; topo II, topoisomerase II; THBA, trihydroxybenzoate.

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inhibitors have achieved more limited clinical utility, primarily in reducing the cardiotoxicity associated with doxorubicin administration [9–11].

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 [12,13]. Salicylate blocks topo II ATPase activity through a non-competitive mechanism, which we have determined occurs secondary to interfering with DNA cleavage [13]. 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 [12]. Critically, pretreatment of cells with salicylate prevents doxorubicin-induced DNA double-stranded breaks and reduces the efficacy of both doxorubicin and etoposide in cyto [12].

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 [14]. Now common for its daily use in the prevention of cardiovascular and cerebrovascular events, in addition to being used for its anti-inflammatory, antipyretic and analgesic effects, it is estimated that almost 40,000 metric tons of aspirin are ingested annually worldwide [15]. Many compounds found in edible plants and spices bear structural similarity to salicylate [16,17] and diets rich in these foods can significantly increase plasma levels of salicylate [17]. 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 [12]. 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.

2. Materials and methods

2.1. 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.

2.2. 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 [18]. Electrophoresis and immunoblotting conditions were also as previously described [12].

2.3. 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 [12].

2.4. Kinetoplast DNA (kDNA) decatenation assay

The effects of the salicylate-related analogs on topo IIα catalytic activity were examined as previously described [12], with the following modifications. Human topo IIα (2 U) was incubated with 200 ng kinetoplast DNA (kDNA, Topogen) in reaction buffer A

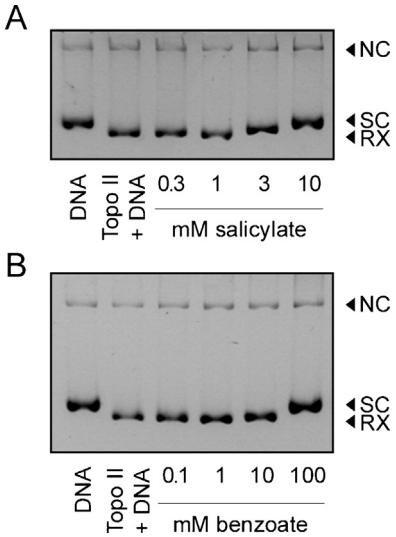

Fig. 1. Salicylate is a more potent inhibitor of topo II catalytic activity than benzoate. The ability of purified topo II (8 U) to relax supercoiled pBR322 plasmid DNA was examined in vitro in the presence of salicylate (A) or benzoate (B). Reactions were incubated for 10 min on ice prior to the addition 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.