

Aspirin metabolites 2,3-DHBA and 2,5-DHBA inhibit cancer cell growth: Implications in colorectal cancer prevention

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Abstract. Although compelling evidence exists on the ability of aspirin to treat colorectal cancer (CRC), and numerous theories and targets have been proposed, a consensus has not been reached regarding its mechanism of action. In this regard, a relatively unexplored area is the role played by aspirin metabolites 2,3-dihydroxybenzoic acid (2,3-DHBA) and 2,5-dihydroxybenzoic acid (2,5-DHBA) in its chemopreventive actions. In a previous study, we demonstrated that 2,3-DHBA and 2,5-DHBA inhibited CDK1 enzyme activity *in vitro*. The aim of the present study was to understand the effect of these metabolites on the enzyme activity of all CDKs involved in cell cycle regulation (CDKs 1, 2, 4 and 6) as well as their effect on clonal formation in three different cancer cell lines. Additionally, *in silico* studies were performed to determine the potential sites of interactions of 2,3-DHBA and 2,5-DHBA with CDKs. We demonstrated that 2,3-DHBA and 2,5-DHBA inhibits CDK-1 enzyme activity beginning at 500 μ M, while CDK2 and CDK4 activity was inhibited only at higher concentrations (>750 μ M). 2,3-DHBA inhibited CDK6 enzyme activity from 250 μ M, while 2,5-DHBA inhibited its activity >750 μ M. Colony formation assays showed that 2,5-DHBA was highly effective in inhibiting clonal formation in HCT-116 and HT-29 CRC cell lines (250-500 μ M), and in the MDA-MB-231 breast cancer cell line (~100 μ M). In contrast 2,3-DHBA was effective only in MDA-MB-231 cells (~500 μ M). Both aspirin

and salicylic acid failed to inhibit all four CDKs and colony formation. Based on the present results, it is suggested that 2,3-DHBA and 2,5-DHBA may contribute to the chemopreventive properties of aspirin, possibly through the inhibition of CDKs. The present data and the proposed mechanisms should open new areas for future investigations.

Introduction

Aspirin which was synthesized and patented in 1897 for its analgesic and anti-inflammatory properties has been used now for more than a century, and is one of the most widely used drugs in medicine (1). Its mechanism of action was established in 1971 when Sir John Vane in England demonstrated its ability to decrease the synthesis of prostaglandins responsible for pain, inflammation and fever (2). In 1975, it was discovered that aspirin decreases prostaglandin synthesis by acetylating and inactivating cyclooxygenases (COX) (3). In the years that followed thereafter, aspirin was recommended for the prevention of secondary cardiovascular diseases such as myocardial infarction and stroke, due to its ability to decrease thromboxane A2 in platelets (4). Studies conducted in the past three decades have established yet another therapeutic use for aspirin in the prevention of epithelial cancers, particularly colorectal cancer (CRC), and this was observed even with the low dose aspirin (75 mg) (5-7). Owing to the growing global prevalence of cancer, the chemo-preventive effects of aspirin has gained much attention among physicians, scientists and the public alike. In fact, in 2016 the United States preventive services task force (USPSTF) recommended 'initiating low dose aspirin use for the prevention of cardiovascular disease and CRC in adults aged 50-59 years' (8). This recommendation was based on increasing evidences on low-dose aspirin's ability to decrease CRC and other cancers (9,10).

Though numerous reports have described potential mechanisms of cancer-prevention by aspirin, its primary mode of action still remains elusive. One of the most discussed mechanism involves the inhibition of COX in platelets (platelet hypothesis) (9-14). Independent of this, other mechanisms have been proposed that includes inhibition of NF- κ B (15), induction of polyamine catabolism (16), activation of AMP-kinase (17,18), inhibition of Wnt signaling and

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Abbreviations: 2,3-DHBA, 2,3-dihydroxybenzoic acid; 2,5-DHBA, 2,5-dihydroxybenzoic acid

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β -catenin phosphorylation (19), downregulation of c-myc (20), induction of DNA mismatch repair proteins (21), induction of autophagy (22), and acetylation of p53 and glucose-6-phosphate dehydrogenase (23,24) to name a few. Many of these effects were detected mainly at high concentrations of aspirin (often at millimolar concentrations) which are not reached *in vivo* in the systemic circulation (maximum reported concentration is 142 μ M with 1.2 g tablets/4-6h) (16). The inability to accurately pinpoint the mechanism involved also stems from the failure to differentiate the primary proximal effects from its associated downstream signaling events and the subsequently observed biological responses (25). Additionally, studies show that aspirin is more effective in decreasing the incidence of CRC as compared to other cancers such as breast, prostate, lung and skin (26-28); however, it is still not clear why aspirin is more effective against CRC as compared to other cancers.

Our laboratory has been focusing on determining a role for aspirin and salicylic acid metabolites 2,3-dihydroxybenzoic acid (2,3-DHBA) and 2,5-dihydroxybenzoic acid (2,5-DHBA), known to be generated in the body by the cytochrome P-450 (CYP450) catalyzed reactions (29), in the prevention of CRC. Interestingly, similar dihydroxybenzoic acids have also been reported to be generated from aspirin by the gut microflora (30). In a previous study we demonstrated the ability of 2,3-DHBA and 2,5-DHBA to inhibit cyclin dependent kinase 1 (CDK1) activity *in vitro* (31). However, an extended study on the effect of these metabolites on other CDKs involved in cell-cycle regulation (CDK2, CDK4 and CDK6) as well as the demonstration of their ability to inhibit cancer cell growth were not reported. As a dysregulated cell cycle is one of the hallmarks of cancer, it was important to determine if 2,3-DHBA and 2,5-DHBA affected cancer cell growth by inhibiting these CDKs to gain a comprehensive understanding of their mechanism of action. In the present study, we investigated the effect of 2,3-DHBA and 2,5-DHBA on CDKs 1, 2, 4 and 6 enzyme activities and determined their potential sites of interaction within these enzymes. In addition, we also performed studies to determine the effect of these metabolites on cancer cell proliferation. Our results show that although aspirin and salicylic acid showed potential interactions with the CDK enzymes, only their metabolites were effective in inhibiting CDK enzyme activity and cancer cell proliferation. More specifically, these metabolites inhibited CDK1 and CDK6 enzyme activity. Our results show that among the two aspirin metabolites, 2,5-DHBA is highly effective in inhibiting cell proliferation in three different cell lines (HCT-116, HT-29 and MDA-MB-231) whereas 2,3-DHBA was capable of inhibiting cell growth only in MDA-MB-231 cells. Our findings suggests a role for these metabolites in aspirin's chemo-preventive actions.

Materials and methods

Cell lines, recombinant proteins and reagents. HCT-116 (colorectal carcinoma), HT-29 (colorectal adenocarcinoma) and MDA-MB-231 (breast adenocarcinoma) cell lines were purchased from American Type Culture Collection (ATCC). MDA-MB-231 cells were grown in RPMI media while HCT-116 and HT-29 cells were cultured in McCoy's 5A media, both supplemented with 10% FBS and antibiotics for 24 h before treatment with specified compounds for indicated

times. Authentication of cell lines was done by ATCC through their DNA-STR profile. CDK1/Cyclin B1, CDK2/Cyclin A2, CDK4/Cyclin D1, Retinoblastoma (C-term) and kinase buffer were purchased from SignalChem. Aspirin, salicylic acid and trypsin-EDTA solution were purchased from Sigma-Aldrich; H1 Histones were obtained from EMD Millipore; [32 P] γ -ATP from PerkinElmer; 2,3-DHBA, 2,5-DHBA and all other reagents mentioned in this study were obtained from Thermo Fischer Scientific, Inc.

In vitro CDK assay. *In vitro* CDK assays, to measure enzyme activity, were performed as described by the manufacturer (32-35) and previously published protocols (31,36,37). Briefly, purified enzyme was aliquoted into the reaction buffer and incubated with indicated compounds (aspirin, salicylic acid, 2,3-DHBA and 2,5-DHBA) at various concentrations for 10 min. at room temperature. The reaction mixture was incubated with kinase buffer containing 15 μ M ATP, 2 μ Ci of [32 P] γ -ATP, 5 μ g of H1 Histone (7.5 μ g/reaction added as substrate for CDKs 1 and 2) or retinoblastoma (1.5 μ g/reaction added as substrate for CDKs 4 and 6), at 30°C for 20 min in a final volume of 50 μ l. The reactions were halted by adding EDTA to a final concentration of 20 mM and addition of 4X loading buffer. The samples were boiled for 10 min, analyzed by 8 or 10% SDS-PAGE, stained using coomassie brilliant blue (R250) to confirm equal loading of the H1 histones and Retinoblastoma protein. The molecular weight of the proteins were confirmed by molecular weight markers. The gel was dried, and exposed to X-ray film. NIH ImageJ software was used to quantify the intensities of the bands. The normalization of the band intensities of the phosphorylated H1 histones/ Retinoblastoma proteins were determined by comparing to the control, which is the phosphorylation in the absence of these compounds.

Molecular docking studies. The crystallographic three dimensional structures of CDK1 (4Y72 A chain), CDK2 (1FIN A chain), CDK4 (3G33 A chain) and CDK6 (4AUA) were retrieved from the Protein Data Bank (PDB). Energy minimization for these proteins was performed using Gromacs 3.3.1 package utilizing GROMOS96 force field (38). The energy-minimized molecules were used as the receptors for virtual small molecule docking with aspirin, salicylic acid, 2,3-DHBA, 2,5-DHBA using AutoDock Vina. The results were visualized by PYMOL molecular graphics system version 1.3.

Cell proliferation. Approximately 250,000 cells were seeded per 100 mm plates containing 10% FBS and grown overnight. Cells were left untreated (control) or were treated with drugs at various concentrations and incubated for 48 h. The floating cells in the spent media (if any) were collected, pooled with the trypsinized cells, and counted using the Nexcelom Cellometer Auto T4 cell counter.

Clonogenic assay. Clonogenic assays were performed as previously described (36). Cells were seeded at a density of 500 cells/100 mm plate and grown for 48 h following which cells were left untreated (control) or treated with compounds (aspirin, salicylic acid, 2,3-DHBA and 2,5-DHBA) at the concentrations indicated. The spent media was replaced

CDK1

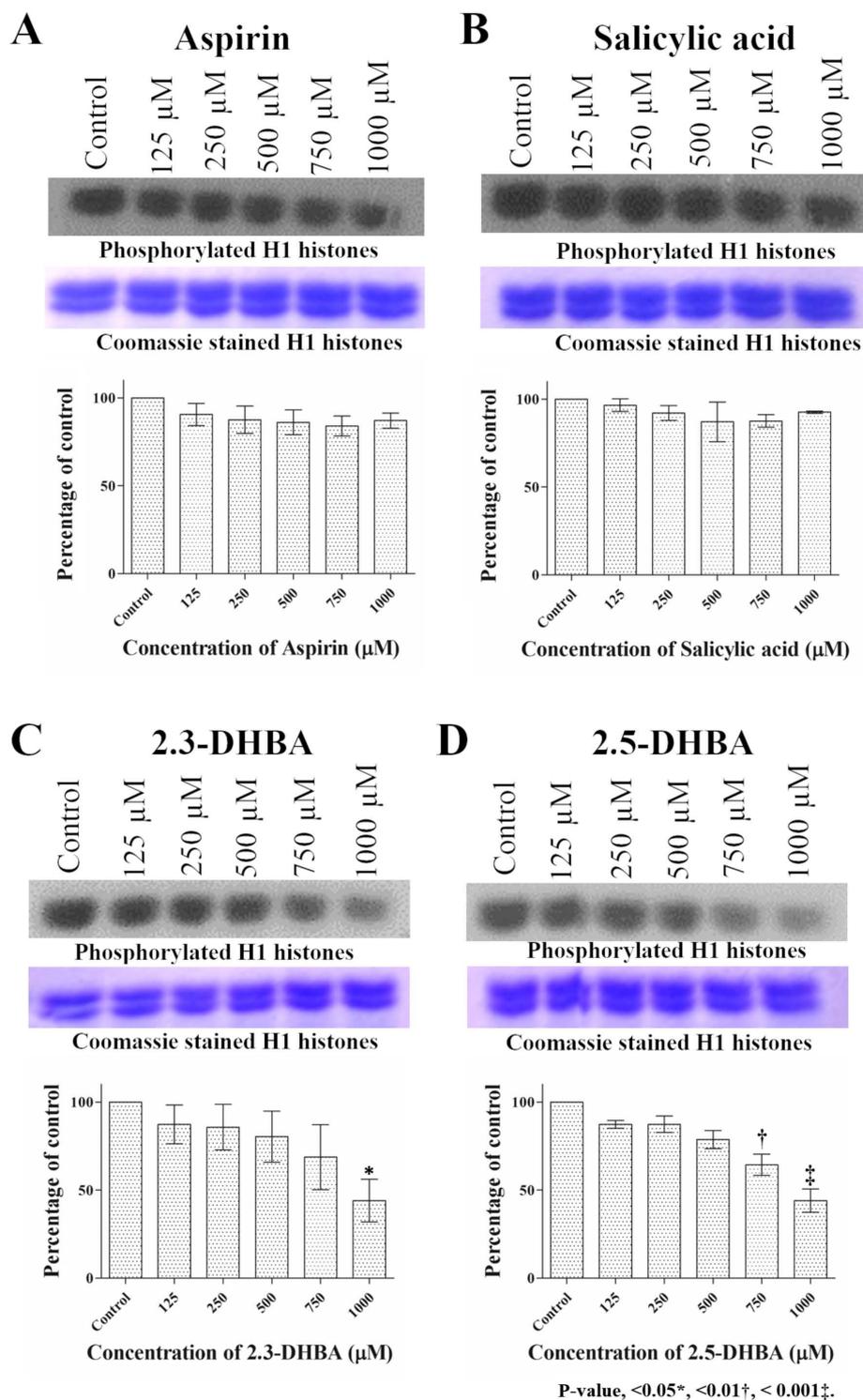


Figure 1. Effect of different concentrations of (A) aspirin, (B) salicylic acid, (C) 2,3-DHBA and (D) 2,5-DHBA on CDK1 enzyme activity. H1 histones were used as the substrate for CDK1. Upper panel shows phosphorylation of H1 Histones in the presence of different concentrations of each compound. The middle panel shows the coomassie blue stained proteins. Lower panel represents the quantification of the bands in upper panel. *P<0.05, **P<0.01, ***P<0.001 vs. Control. 2,3-DHBA, 2,3-dihydroxybenzoic acid; 2,5-DHBA, 2,5-dihydroxybenzoic acid.

with fresh media containing the respective compounds every 5-6 days. Cells were incubated for 21 days, fixed with 100% methanol for 20 min, and stained with 0.5% crystal violet prepared in 25% methanol. The colonies were then photographed and quantified using NIH ImageJ software.

Statistical analysis. All experiments were repeated 3-6 times independently of each other. One-way ANOVA followed by Tukey's post-hoc tests were adopted to compare group differences with the control. P<0.05 was considered to indicate a statistically significant difference.

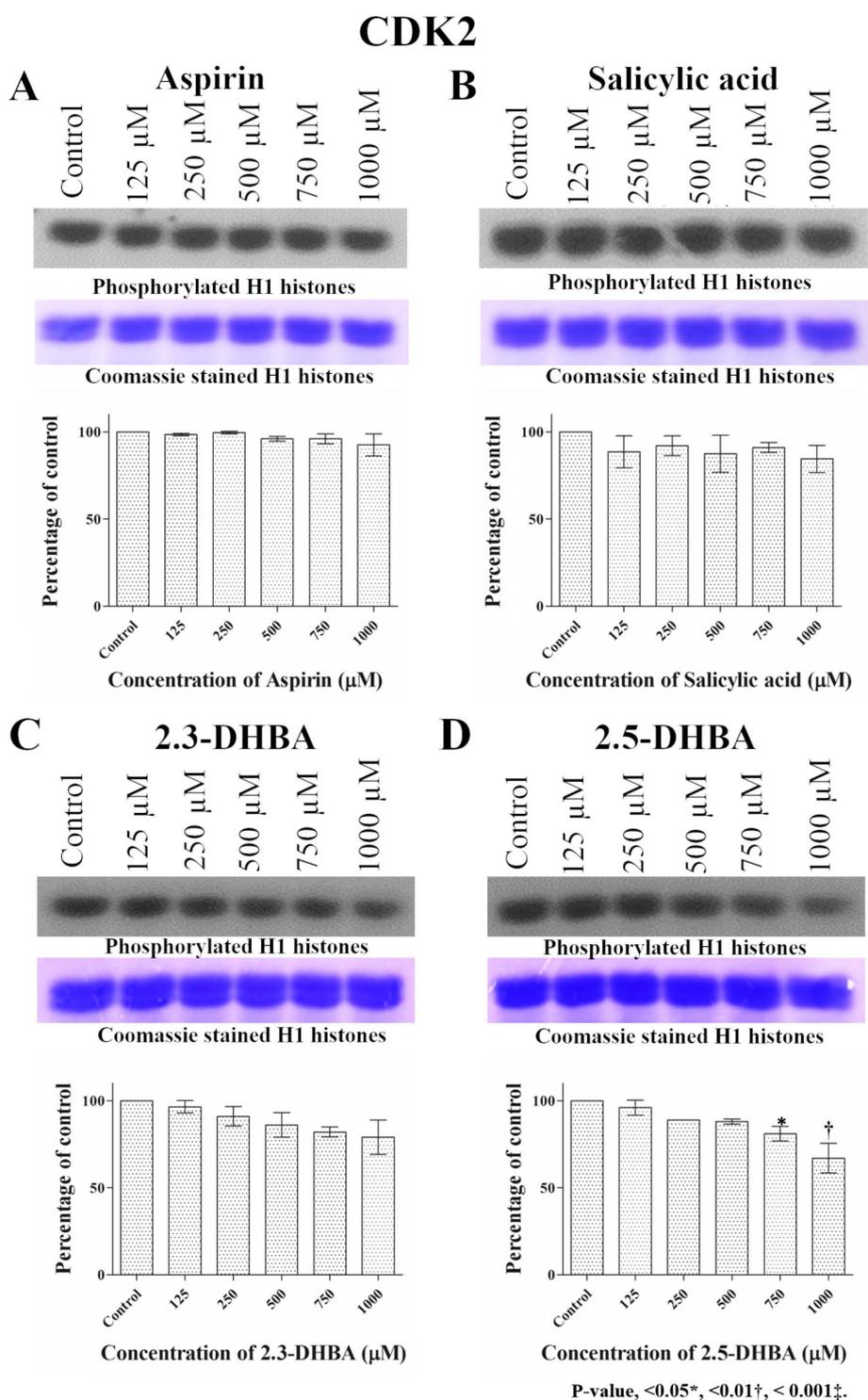


Figure 2. Effect of different concentrations of (A) aspirin, (B) salicylic acid, (C) 2,3-DHBA and (D) 2,5-DHBA on CDK2 enzyme activity. H1 histones were used as the substrate for CDK2. Upper panel shows phosphorylation of H1 Histones in the presence of different concentrations of each compound. The middle panel shows the coomassie blue stained proteins. Lower panel represents the quantification of the bands in upper panel. *P<0.05, **P<0.01 vs. Control. 2,3-DHBA, 2,3-dihydroxybenzoic acid; 2,5-DHBA, 2,5-dihydroxybenzoic acid.

Results

Effect of aspirin, salicylic acid, 2,3-DHBA and 2,5-DHBA on CDK enzyme activity. We initiated this study by determining the dose-dependent effect of aspirin, salicylic acid, 2,3-DHBA and 2,5-DHBA on recombinant CDKs 1, 2, 4 and 6 by measuring the phosphorylation pattern through *in vitro*

kinase assays. Fig. 1 shows that CDK1 activity remained unaffected following treatment with aspirin and salicylic acid at all concentrations tested (125 μM to 1 mM); however, 2,3-DHBA and 2,5-DHBA inhibited CDK1 activity, confirming our previously published results (31). In both cases, inhibition was around 20% at 500 μM, 40% at 750 μM and 60% at 1,000 μM. CDK2 activity remained unchanged upon exposure to

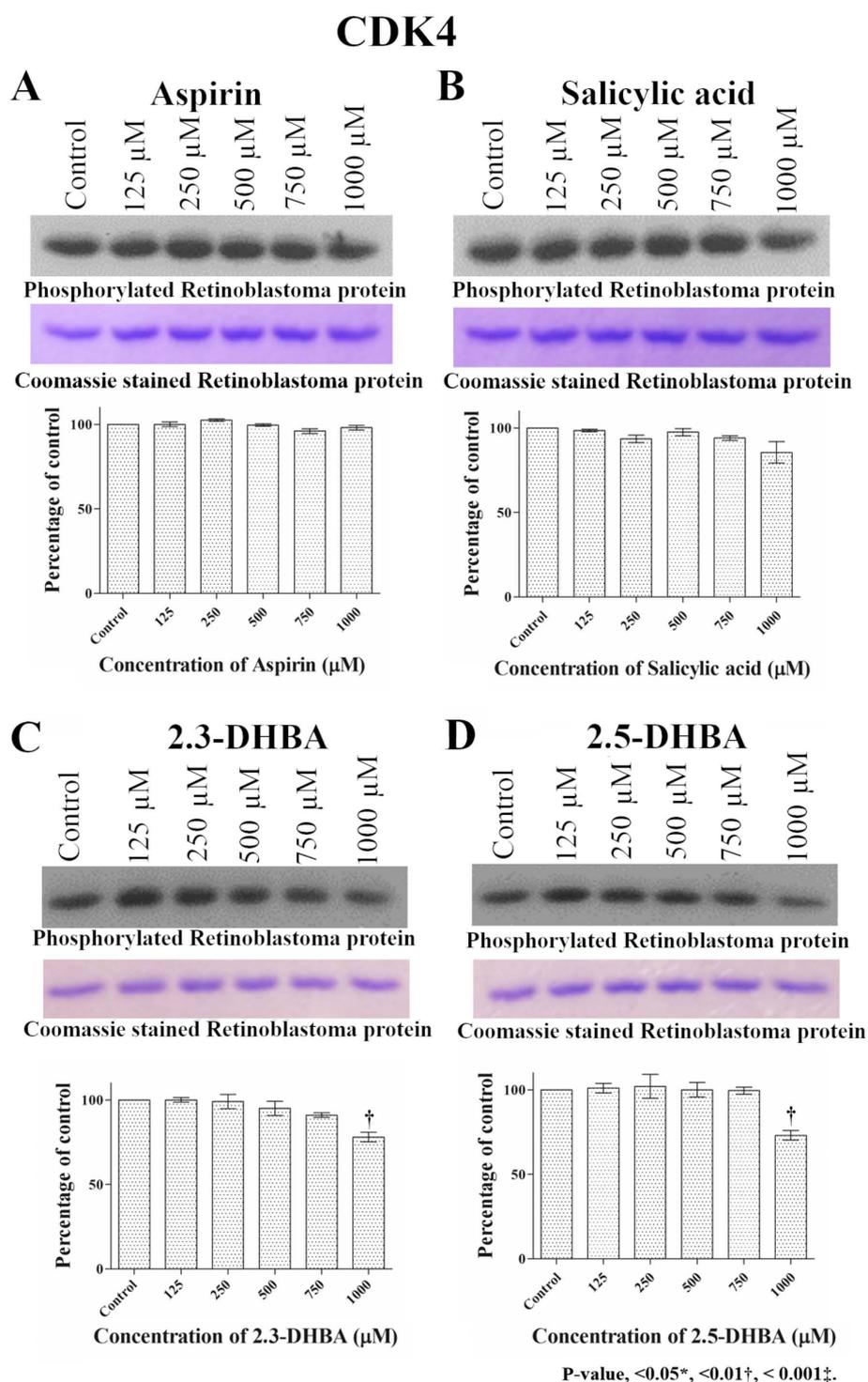


Figure 3. Effect of different concentrations of (A) aspirin, (B) salicylic acid, (C) 2,3-DHBA and (D) 2,5-DHBA on CDK4 enzyme activity. Retinoblastoma protein was used as the substrate for CDK4. Upper panel shows phosphorylation of retinoblastoma protein in the presence of different concentrations of each compound. The middle panel shows the coomassie blue stained proteins. Lower panel represents the quantification of the bands in upper panel. **P<0.01 vs. Control. 2,3-DHBA, 2,3-dihydroxybenzoic acid; 2,5-DHBA, 2,5-dihydroxybenzoic acid.

aspirin and salicylic acid while inhibition was observed with 2,3-DHBA and 2,5-DHBA, especially at higher concentrations (>750 μM ; Fig. 2). Similar to CDK1 and CDK2, aspirin and salicylic acid both failed to inhibit CDK4 and CDK6 enzyme activity (Figs. 3 and 4). 2,3-DHBA and 2,5-DHBA inhibited CDK4 activity at 1,000 μM . 2,3-DHBA was able to inhibit CDK6 enzyme activity beginning at 250 μM , while 2,5-DHBA

inhibited CDK6 enzyme activity at concentrations >750 μM . These results show 2,3-DHBA and 2,5-DHBA were able to inhibit CDKs 1, 2, 4, and 6 activities to different degrees. It is also important to note that the inhibition observed with CDK1 by 2,3-DHBA and 2,5-DHBA, and CDK-6 by 2,3-DHBA was significantly greater compared to inhibition of other CDKs by these compounds.

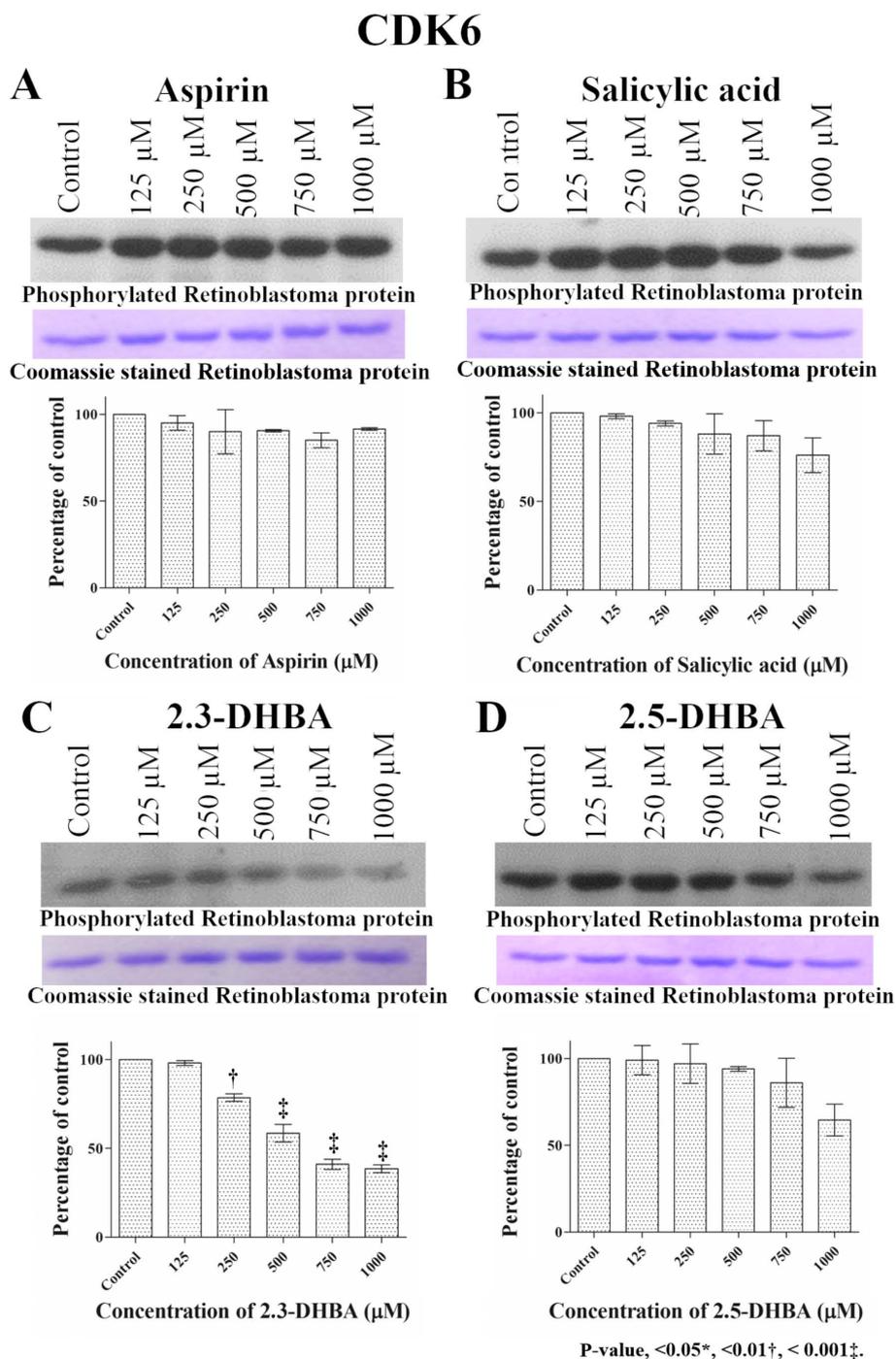


Figure 4. Effect of different concentrations of (A) aspirin, (B) salicylic acid, (C) 2,3-DHBA and (D) 2,5-DHBA on CDK6 enzyme activity. Retinoblastoma protein was used as the substrate for CDK6. Upper panel shows phosphorylation of retinoblastoma protein in the presence of different concentrations of each compound. The middle panel shows the coomassie blue stained proteins. Lower panel represents the quantification of the bands in upper panel. **P<0.01, ***P<0.001 vs. Control. 2,3-DHBA, 2,3-dihydroxybenzoic acid; 2,5-DHBA, 2,5-dihydroxybenzoic acid.

Docking studies reveal binding pockets for aspirin, salicylic, 2,3-DHBA, and 2,5-DHBA. AutoDock Vina was used to predict the potential interactions of aspirin, salicylic acid, 2,3-DHBA and 2,5-DHBA with CDKs 1, 2, 4 and 6. The binding free energy and hydrogen bond lengths were also determined. The results of the docking studies are shown in Table I and Fig. 5 (line model). All the compounds tested interacted with CDKs and utilized amino acids either residing in the active site or at alternate binding pockets. Docking studies revealed that 2,3-DHBA potentially interacts with CDK1 through Asp146;

with CDK2 it utilizes Lys33, and Asp145; with CDK4, it interacts through Cys68 and His158 and with CDK6 it binds to Asp134, Tyr292, Ala291 and Thr198. 2,5-DHBA interacts with CDK1 using Asp146; with CDK2 it utilizes Asp145 and Lys33; with CDK4 through His158; and with CDK6 it uses Lys43, Tyr24 and Gly25. Salicylic acid and aspirin also interacted with all 4 CDKs (Table I), although CDK inhibition was not observed in vitro (Figs. 1-4). Notable interactions are that between the drug molecules and Asp146 of CDK1, as this amino acid is involved in CDK-ATP interactions (39).

Table I. Free energy binding values and hydrogen bond lengths for the interaction of aspirin, salicylic acid, 2,3-DHBA and 2,5-DHBA with CDK1, CDK2, CDK4 and CDK6.

A, CDK1				
Ligand	Interacting amino acids	Measurement, Å°	No of H bonds	Energy value, kCal/mol
Aspirin	Asn133, Tyr15	2.5, 3.0	2	-6.6
Salicylic acid	Asp146, Lys33	2.8, 3.5	2	-6.4
2,3-DHBA	Asp146	2.9	1	-5.9
2,5-DHBA	Asp146	2.9	1	-5.9
B, CDK2				
Ligand	Interacting amino acids	Measurement, Å°	No of H bonds	Energy value, kCal/mol
Aspirin	Asp145, Glu51, Lys33(2)	3.2, 2.6, 3.1, 3.0	4	-6.2
Salicylic acid	Asp145, Lys33(2)	3.3, 3.0, 3.3	3	-5.7
2,3-DHBA	Lys33, Asp145	3.2, 3.2	2	-5.6
2,5-DHBA	Asp145, Lys33(2)	3.2, 2.9, 2.9	3	-5.9
C, CDK4				
Ligand	Interacting amino acids	Measurement, Å°	No of H bonds	Energy value, kCal/mol
Aspirin	His158, Gln183	2.9, 2.0	2	-5.8
Salicylic acid	His158, Gln183	2.8, 1.8	2	-5.4
2,3-DHBA	His158, Cys68	3.1, 2.2	2	-5.8
2,5-DHBA	His158	3.1	1	-5.4
D, CDK6				
Ligand	Interacting amino acids	Measurement, Å°	No of H bonds	Energy value, kCal/mol
Aspirin	Lys43	2.9	1	-5.4
Salicylic acid	Gly165, Leu166, Arg144	3.1, 2.8, 3.0	3	-5.1
2,3-DHBA	Asp134, Tyr292, Ala291(2), Thr198	2.0, 3.0, 3.2, 2.9, 2.9	5	-5.4
2,5-DHBA	Lys43(2), Tyr24, Gly25	3.2, 3.2, 3.2, 3.2	4	-5.3

2,3-DHBA, 2,3-dihydroxybenzoic acid; 2,5-DHBA, 2,5-dihydroxybenzoic acid.

Although salicylic acid interacted with CDK1 through Asp146 and Lys33 and CDK2 through Asp145 and Lys33, it is not clear at this stage why it failed to cause inhibition of the enzyme activity. This may be due to the formation of an intramolecular hydrogen bond between its carboxyl and hydroxyl groups which may hinder its interactions with CDKs.

Effect of aspirin, salicylic acid, 2,3-DHBA and 2,5-DHBA on colony formation. Clonogenic assay was performed to determine the effectiveness of aspirin, salicylic acid, 2,3-DHBA and 2,5-DHBA on colony formation in HCT-116 and HT-29 colon cancer cells as well as MDA-MB-231 breast cancer cells. HCT-116 and HT-29 cells were chosen to study the effect of these compounds as these cell line have been extensively used in studies pertaining to aspirin's biological effects (40,41);

MDA-MB-231 cells were chosen to determine if this effect was mimicked in another cancer cell line. We observed that aspirin and salicylic acid both failed to inhibit colony formation in HCT-116 (Fig. 6A and B), HT-29 (Fig. 7A and B) and MDA-MB-231 cells (Fig. 8A and B). 2,3-DHBA was effective in MDA-MB-231 cells at higher concentrations (>500 µM) (Fig. 8C), while it was ineffective in HCT-116 (Fig. 6C) and HT-29 cells (Fig. 7C). Interestingly, 2,5-DHBA exhibited a dose dependent effect on colony formation in all cell lines examined (Figs. 6D, 7D, 8D). In HCT-116 cells, decreased colony formation was observed with 2,5-DHBA at concentrations >250 µM, in HT-29 cells at concentrations >200 µM, and in MDA-MB-231 cells, beginning at 50 µM. In contrast, two other salicylic acid derivatives 2,4-DHBA and 2,6-DHBA were unable to inhibit colony formation in

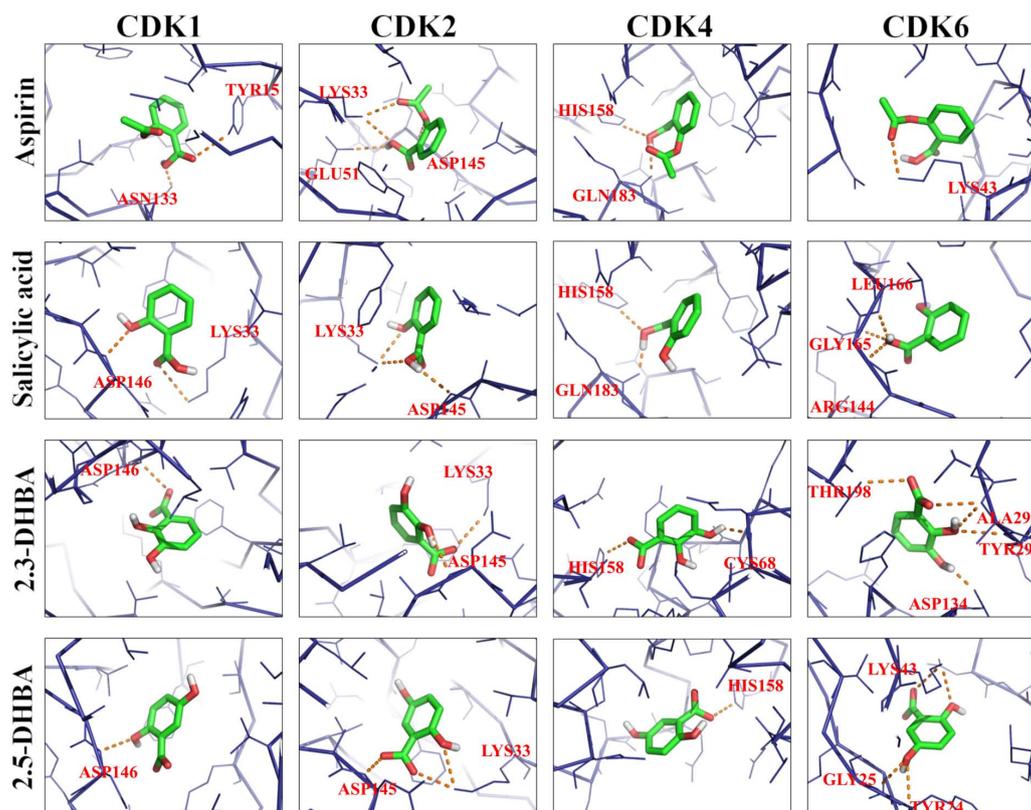


Figure 5. Line model showing the interactions of aspirin, salicylic acid, 2,3-DHBA and 2,5-DHBA with CDK1, CDK2, CDK4 and CDK6.

MDA-MB-231 cells (Fig. S1). These results clearly show that although the sensitivity varies among different cell lines, both 2,3-DHBA and 2,5-DHBA exhibit the ability to inhibit cancer cell proliferation in contrast to aspirin and salicylic acid.

Interestingly, 2,3-DHBA and 2,5-DHBA were ineffective in inhibiting cell proliferation in MDA-MB-231 cells over a range of concentrations (125-1,000 μM) for 24-48 h (Fig. S2). We suggest that the lack of inhibition in acute treatment observed may be related to the poor uptake of these compounds.

Discussion

Despite the growing evidence for aspirin's ability to decrease CRC, the mechanisms involved remain poorly defined. Numerous hypotheses commencing from the COX inhibition in platelets (9,11,12) to increased expression of DNA repair proteins, transcription factors and interconnected signaling pathways have been proposed (42-45); however, a consensus has not been reached regarding the target/pathway(s) primarily responsible for its anti-cancer effects (46). In this research paper, we tested the hypothesis that aspirin metabolites 2,3-DHBA and 2,5-DHBA may mediate its chemopreventive actions by inhibiting CDKs and affecting cell growth. We provide evidence that 2,3-DHBA and 2,5-DHBA inhibit CDKs involved in cell-cycle regulation (CDKs 1, 2, 4 and 6), although to different degrees. Inhibition of CDK1 by 2,3-DHBA and 2,5-DHBA was observed at 500 μM , and CDK-6 by 2,3-DHBA was observed starting at 250 μM , however, inhibition of other CDKs by these compounds required much higher concentrations (750 μM), suggesting that CDK1 and CDK6 are better

targets for 2,3-DHBA and/or 2,5-DHBA. In contrast, aspirin and salicylic acid both failed to inhibit CDK-1, 2, 4 and 6 enzyme activity. Through molecular docking studies we identified potential sites of these interactions; most of the interacting amino acids appear to be localized in the active site of these enzymes. Importantly, we also demonstrated the ability of aspirin/salicylic acid metabolites to inhibit clonal formation in cancer cell lines. 2,5-DHBA inhibited colony formation in a dose-dependent manner in three different cancer cell lines (HCT-116, HT-29 and MDA-MB-231). In contrast, 2,3-DHBA exhibited effective inhibition in MDA-MB-231 cells alone. These results suggest that 2,3-DHBA and 2,5-DHBA may be involved in aspirin's chemopreventive actions. Our findings raise two important questions: 1) What are the sources of the aspirin metabolites 2,3-DHBA and 2,5-DHBA?; 2) Are there unique scenarios that gives an anatomical advantage to 2,3-DHBA and 2,5-DHBA to act against CRC?.

Plain aspirin (non-enteric coated) is absorbed in the stomach where the acidic environment protects it from deacetylation and ionization, resulting in its faster absorption; however, aspirin may also be absorbed in the upper small intestine and enter into the hepatic portal vein (47-49). In addition, lymphatic uptake of some aspirin and salicylic acid has been reported (49). We hypothesize that aspirin's preferential effect against CRC (over other tissues) may be related to the generation of salicylic acid that occurs both through hydrolysis and the action of gut esterases (50) in the intestine or colon. Intact aspirin's bioavailability is between 50-68% (51,52), and it is estimated that nearly 29-39% of aspirin may be hydrolyzed in the GI tract, particularly in the duodenum and ileum (52).

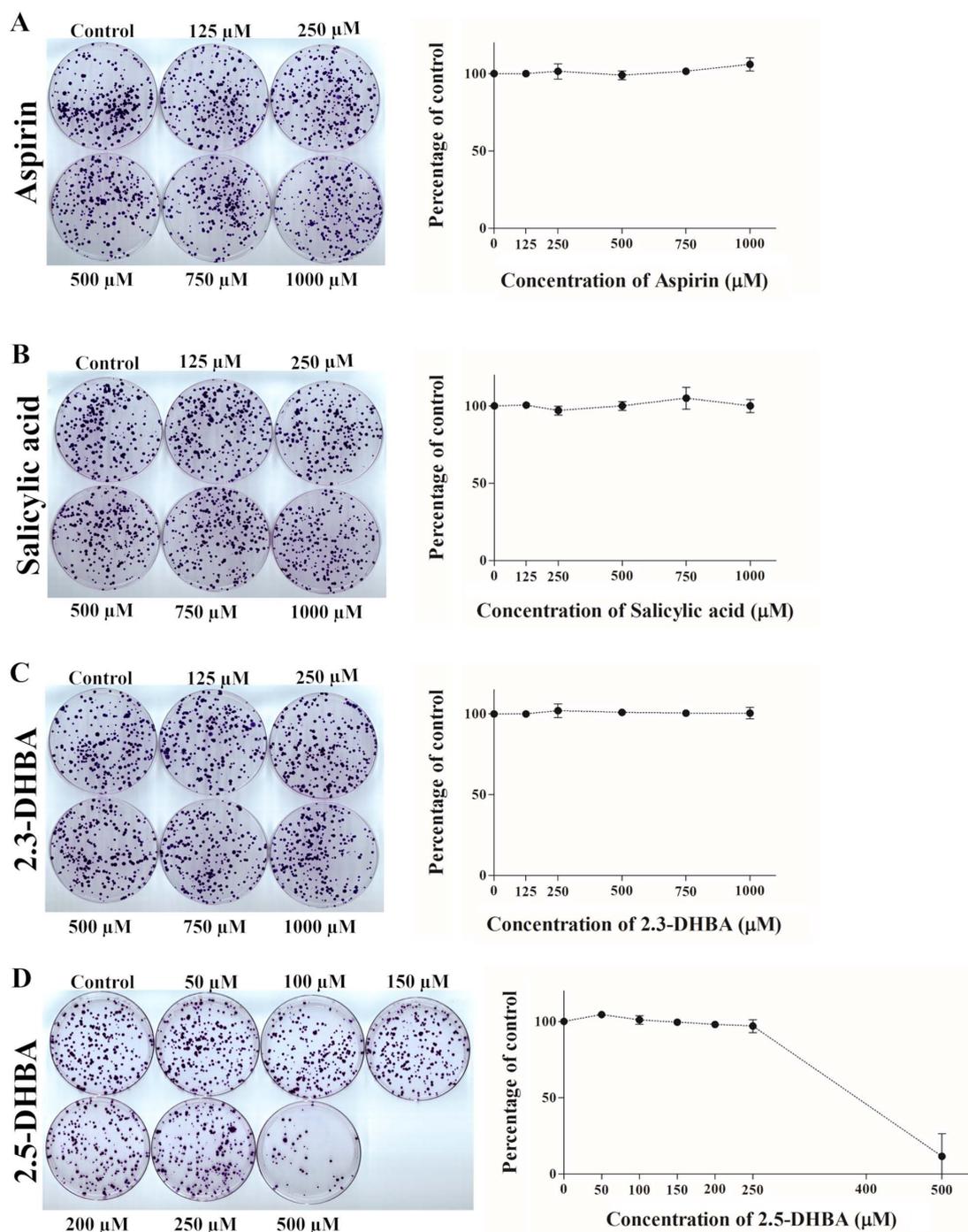


Figure 6. Effect of (A) aspirin, (B) salicylic acid, (C) 2,3-DHBA and (D) 2,5-DHBA on colony formation in HCT-116 cells. Quantification of the data is shown beside the crystal violet stained image of the colonies. The graph is represented as the mean \pm standard deviation. 2,3-DHBA, 2,3-dihydroxybenzoic acid; 2,5-DHBA, 2,5-dihydroxybenzoic acid.

Therefore, it can be argued that epithelial cells of the intestine and colon are likely to be exposed to substantially higher concentrations of salicylic acid compared to other tissues in the body. With enteric coated aspirin, the concentration of aspirin/salicylic acid exposed to the GI cells may be even greater than non-enteric coated tablets as the bioavailability of intact aspirin from enteric coated tablets is significantly lower compared to plain aspirin due to slow release and absorption in the intestine (47,53).

Aspirin in the systemic circulation is known to be metabolized in the liver to 2,3-DHBA and 2,5-DHBA through the

action of cytochrome P450 (CYP450s) enzymes. However, it is unlikely that these liver generated metabolites contribute to the anti-cancer effect in colorectal tissues due to their low concentrations in the plasma (29). We suggest that there are two routes through which intestinal epithelial cells may get exposed to 2,3-DHBA and 2,5-DHBA. As intestinal epithelial cells also express CYP450s similar to the isoforms expressed in the liver involved in aspirin metabolism (54), one would expect aspirin metabolites to be generated in these cells; however, it is not clear at this stage to what extent these metabolites are produced within the GI cells. Since exposure of HCT-116,

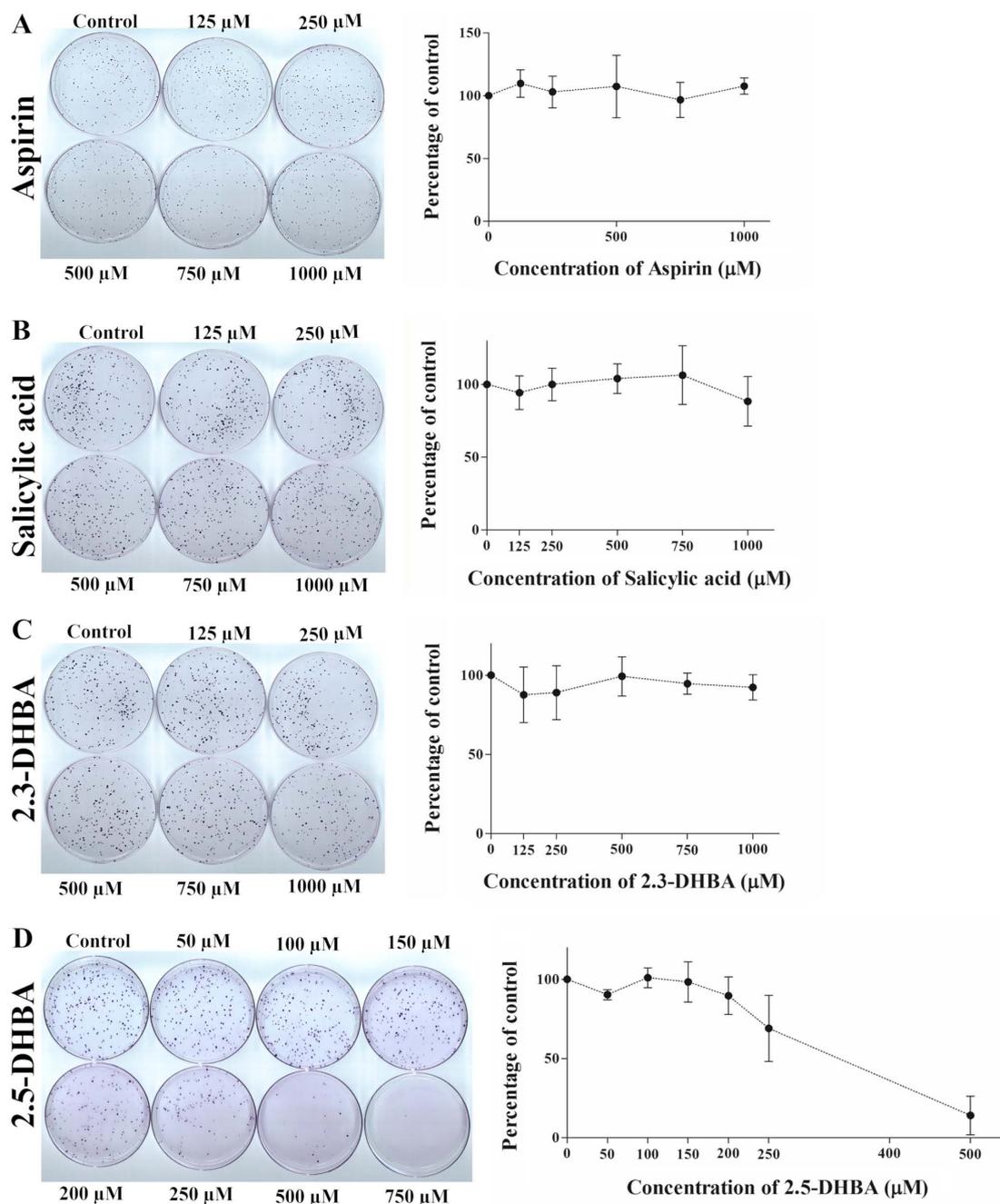


Figure 7. Effect of (A) aspirin, (B) salicylic acid, (C) 2,3-DHBA and (D) 2,5-DHBA on colony formation in HT-29 cells. Quantification of the data is shown beside the crystal violet stained image of the colonies. The graph is represented as mean \pm standard deviation. 2,3-DHBA, 2,3-dihydroxybenzoic acid; 2,5-DHBA, 2,5-dihydroxybenzoic acid.

HT-29 and MDA-MB-231 cells to aspirin or salicylic acid (up to 1 mM) failed to inhibit clonal formation, it is likely that the contributions of cellular CYP450s to the formation of 2,3-DHBA and 2,5-DHBA is negligible, although a small amount may still be produced through this route. We have not determined the effect of aspirin and salicylic acid on clonal formation at concentrations greater than 1 mM, therefore we are not sure if higher concentrations will have an effect on clonal formation. An alternative possibility and an attractive hypothesis is that the significant amount of aspirin/salicylic acid left unabsorbed (32-50%) in the lumen of the intestine and colon maybe metabolized by the intestinal microflora to generate hydroxyl derivatives of salicylic acid, which may

include both 2,3-DHBA and 2,5-DHBA. Bacteria are capable of metabolizing drugs through hydroxylations (55), and one report in 2016 showed that incubation of aspirin with human fecal suspensions containing microbiota resulted in the degradation of aspirin to salicylic acid and hydroxylated salicylic acids (30). The authors also showed that in rats orally administered with aspirin and ampicillin, the bioavailability of aspirin in the plasma was much higher compared to rats administered with aspirin alone, suggesting that microbiota contributes to the degradation of aspirin in the GI tract. An estimation of the salicylic acid present in the lumen of human gut has not been made, nor has the characterization of the hydroxyl derivatives of salicylic acid produced in the lumen been reported till date.

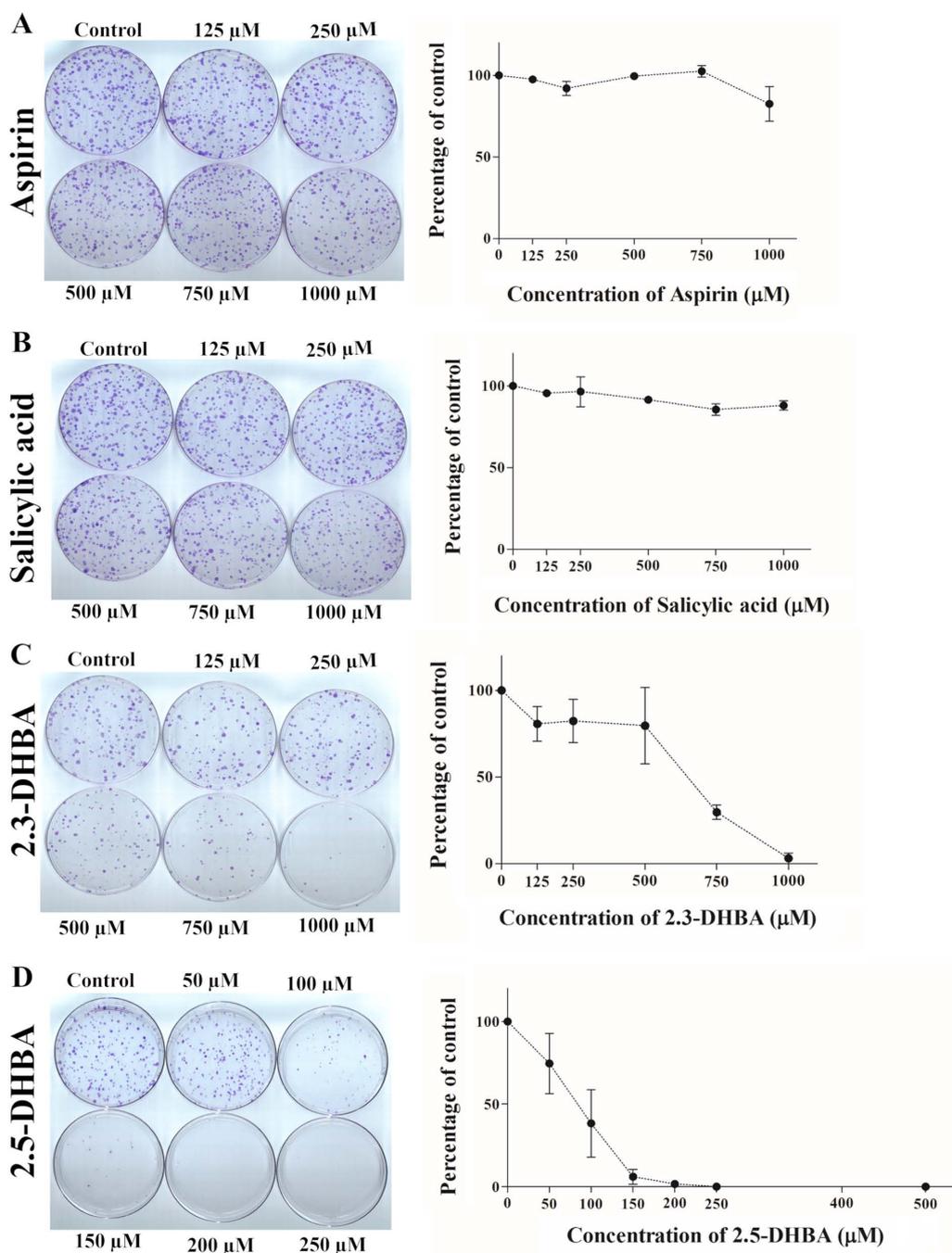


Figure 8. Effect of (A) aspirin, (B) salicylic acid, (C) 2,3-DHBA and (D) 2,5-DHBA on colony formation in MDA-MB-231 cells. Quantification of the data is shown beside the crystal violet stained image of the colonies. The graph is represented as mean \pm standard deviation. 2,3-DHBA, 2,3-dihydroxybenzoic acid; 2,5-DHBA, 2,5-dihydroxybenzoic acid.

The absence of experimental evidence on the potential formation of 2,3-DHBA and 2,5-DHBA, beyond what is reported in literature by Kim *et al* (30), is a limitation of this study. Further work is also required to determine how these metabolites are taken up by the normal, non-cancerous epithelial cells of the intestine and colon and their effect on colony formation, which is also a limitation of this study. Regarding the uptake mechanism, one possibility is that 2,3-DHBA and 2,5-DHBA may be taken up by the monocarboxylate transporter (MCT) SLC5A8 as it is implicated in the transport of other derivatives of salicylic acid (36). The differential sensitivity of HCT-116 and HT-29 vs. MDA-MB-231 could be attributed to the

differential expression of this transporter due to mutational/methylation-driven inactivation of the SLC5A8 gene as previously reported (56).

We observed that 2,5-DHBA was universally effective in inhibiting colony formation as compared to 2,3-DHBA. Significant inhibition of clonal formation was observed with 2,5-DHBA at 500 μ M in HCT-116 cells, at 250 μ M in HT-29 cells and at 100 μ M in MDA-MB-231 cells, whereas 2,3-DHBA failed to inhibit colony formation in HT-29 and HCT-116 cells (125-1,000 μ M). However, it inhibited the colony formation at \sim 500 μ M in MDA-MB-231 cells. It appears that 2,3-DHBA and 2,5-DHBA show differential inhibitory effects

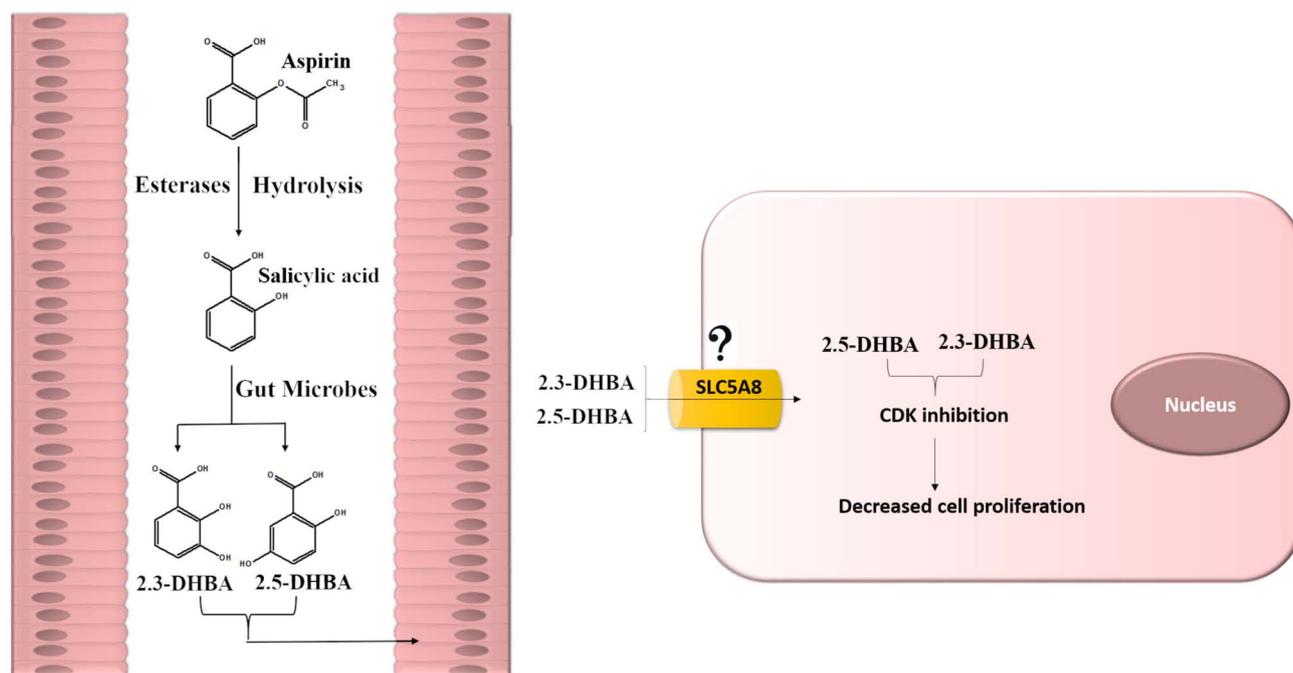


Figure 9. A model depicting how aspirin metabolites may exert its chemopreventive effects on colonic tissue to protect against CRC. It was hypothesized that aspirin and salicylic acid may impede events/pathways crucial to tumor formation through its metabolites 2,3-DHBA and 2,5-DHBA. It was suggested that 2,3-DHBA and 2,5-DHBA generated from salicylic acid by the action of gut microflora is taken up by the colonic epithelial cells. The uptake of these metabolites may occur through SLC5A8, a monocarboxylic acid transporter. Accumulation of these metabolites within the epithelial cells may result in CDK inhibition. This may cause reduced cell proliferation, allowing an opportunity for DNA repair and immune surveillance, thereby preventing tumorigenesis. Alternatively, it is also possible that 2,3-DHBA and 2,5-DHBA may have additional targets contributing to cancer prevention. 2,3-DHBA, 2,3-dihydroxybenzoic acid; 2,5-DHBA, 2,5-dihydroxybenzoic acid.

in different cancer cell lines and it is unclear whether this is due to the differences in uptake of these compounds by the cell lines tested. Two other salicylic acid derivatives namely 2,4-DHBA and 2,6-DHBA failed to inhibit colony formation in MDA-MB-231 cells (Fig. S1). This suggests that among the different salicylic acid derivatives tested, 2,5-DHBA is universally effective whereas 2,3-DHBA is more selective as an anti-proliferative agent. This observation is similar to our previous report in which we showed that another salicylic acid derivative and flavonoid metabolite 2,4,6-THBA is a potent inhibitor of colony formation in cells expressing functional SLC5A8 (36).

In contrast to the inhibitory effect of 2,3-DHBA and 2,5-DHBA in clonal formation (21 days), both compounds failed to show any appreciable inhibitory effect on cell number when cells were treated for 24-48 h (acute treatment) at various concentrations (125 to 1,000 μM ; see Fig. S2). Similarly, in acute treatment experiments, both compounds failed to show an effect on cell cycle progression as measured by flow cytometry, and owing to the lack of an effect, we have not included this data. This discrepancy in the results between acute and chronic treatment may be related to the time required for uptake and accumulation of these compounds to exert an inhibitory effect. We suggest that acute exposure for 24-48 h may result in limited uptake of the drugs resulting in milder effects which are difficult to discern in flow cytometry analysis and cell counting, whereas chronic exposure for 21 days may result in significant accumulation of the drugs sufficient to cause inhibition of cell proliferation as measured by clonal formation assays. As we have not performed flow

cytometry analysis on clonal cells following chronic exposure, it is not clear what stages of the cell cycle are affected by these compounds, and this is another limitation of the study.

Although the IC_{50} of the compounds tested in this study are in micromolar concentrations for both CDK inhibition and clonal formation, it could be argued that it is physiologically relevant in view of their abundance in the intestine due to the hydrolysis of aspirin to salicylic acid in the gut (51,52). It is also important to note that 2,3-DHBA and 2,5-DHBA are abundantly available in fruits and vegetables (57) and one study demonstrated that the phenolic acid content generated from the diet in the gut can rise to micromolar concentrations (58). CDKs and other potential cellular targets probably have evolved to be less sensitive to these compounds to avoid complete inhibition of cell cycle at lower concentrations. The importance of 2,5-DHBA in cancer prevention was also demonstrated in a recent study which showed that it is effective in inhibiting colony formation and DNA synthesis in C6 glioma cells *in vitro*, and enhanced survival of Ehrlich breast ascites carcinoma bearing mice (59). In addition, 2,5-DHBA has been implicated in a previous report as a potential aspirin metabolite formed through the action of tyrosinase enzyme in melanoma cells. The study stated that p-quinone formation from 2,5-DHBA was responsible for aspirin's anti-melanoma effects through ROS formation (60). Thus, although both the studies implicate anti-cancer effects of 2,5-DHBA, the mechanisms described are starkly different; in melanocytes it occurs through depletion of GSH and generation of ROS leading to cell apoptosis (60), while in our study (in HT-29, HCT-116, and MDA-MB-231 cells) it likely involves inhibition of CDKs

leading to suppression of cancer cell growth. This suggests that 2,5-DHBA may contribute to anti-cancer effects through distinct mechanisms in different cell types.

The demonstration of 2,3-DHBA and 2,5-DHBA to inhibit cancer cell growth is a significant observation in view of aspirin's reported ability to decrease CRC. An unanswered question is: What is/are the target(s) for 2,3-DHBA and 2,5-DHBA in cells? Our previously published data (31) and the present study show that 2,3-DHBA and 2,5-DHBA inhibited CDK1/CDK6 activity. However, at this stage it is not known if these two observations are related, or if the inhibition of proliferation by these compounds is due to an alternative mechanism not involving CDKs. In this context, a previous study reported that 2,5-DHBA can interfere with FGF function by disrupting the receptor-growth factor signaling complex *in vitro* and in cell culture studies (61). At this stage, we believe that 2,5-DHBA does not target COX-2 to mediate its chemo-preventive effect in the GI epithelial cells as HCT-116 cells are reported to lack COX-2 expression while HT-29 cells express inactive COX-2 (62). Since both these cell lines were inhibited by 2,5-DHBA, it suggests that the aspirin metabolites can inhibit cancer cell proliferation through a COX-2 independent mechanism. Therefore, greater efforts need to be placed into these relatively unexplored areas to determine how 2,3-DHBA and 2,5-DHBA exert their chemopreventive actions. Whether microbiota generated salicylic acid metabolites becomes available for CDK inhibition following their uptake into the GI cells is an interesting area for future study. If proven, this will highlight the contribution of the gut microflora to aspirin's chemoprevention against CRC, through formation of 2,3-DHBA and 2,5-DHBA. A model depicting how 2,3-DHBA and 2,5-DHBA generated in the GI lumen through the microbial degradation of aspirin/salicylic acid, leading to inhibition of CDKs and cell proliferation is shown in Fig. 9. We suggest that the ability of aspirin to inhibit tumor formation in the intestinal/colonic mucosa may be a local effect via salicylic acid metabolites generated in the gut acting on epithelial cells, and may not require absorption into the blood.

Nearly 35 years have passed since the initial report of aspirin's ability to decrease incidences of CRC (63), however, debates still continue on its mode of action. Although the platelet hypothesis is widely discussed in literature, it has its own short-comings and appears to work indirectly through a set of sequential steps involving two COX isoforms (COX-1 and COX-2) (9-14). COX-1 is the only form of COX in platelets and is constitutively expressed in all tissues, whereas COX-2 is inducible and expressed under inflammatory conditions. COX-2 levels are also elevated in many cancers (48,64-67). The half-life of intact aspirin is about 15-20 min (68) in the systemic circulation and this period appears to be enough to cause inhibition of COX-1 in platelets (1). As chronic inflammation is linked to cancer, it is suggested that aspirin may mediate its effect through inhibition of these COX enzymes. However, aspirin is more effective in the inhibition COX-1 (IC_{50} 1.67 μ M) than COX-2 (IC_{50} 278 μ M) (69). Since low dose aspirin (75-160 mg) is as effective as higher doses (6), the possibility of aspirin directly targeting COX-2 as a potential mechanism to decrease cancer is thought to be unlikely because lower doses administered per day is insufficient

to cause COX-2 inhibition. It is suggested that aspirin's effects may involve sequential inhibition of COX-1 and COX-2 (9,10,12). This theory states that inhibition of COX-1 in platelets leads to prevention of platelet aggregation which subsequently prevents the release of lipid mediators (e.g., PGE-2; TXA2), cytokines and growth factors (e.g., PDGF) from the alpha-granules. These events could inhibit the induction of COX-2 in regions of GI mucosal lesions, preventing tumorigenesis.

If inhibition of COX-1 in platelets in the blood following its absorption is the primary mechanism as proposed in the platelet hypothesis (9-11), one would expect aspirin to decrease the incidences of cancer to the same extent in all tissues in the body, unless one assumes that GI mucosal tissues are more prone to lesions which leads to platelet aggregation, creating an environment suitable for eliciting an inflammatory response. It is intriguing that aspirin that is only present for less than 20 min. in the circulation, while capable of preventing platelet activation and aggregation, could have such a profound effect against CRC development. While inhibition of COX-1 in platelets may play a role (e.g., in the inhibition of metastasis), it is likely that other pathways may play a more significant role in aspirin's cancer preventive actions.

Although the 'platelet hypothesis' is an attractive theory, we suggest that alternative mechanisms as proposed in this study involving locally generated salicylic acid metabolites by the gut microflora from aspirin as potential contributors to chemoprevention should be considered. As ~50% of orally administered aspirin is left unabsorbed in the GI lumen (51,52), one would expect the concentration of aspirin from an 81 mg tablet to be in the range of 0.3 to 1.4 mM in the gut assuming that the total GI fluid volume under fed and fasting conditions are ~750 and ~160 ml respectively (70). Presence of microflora in the GI lumen may thus be able to generate 2,3-DHBA and/or 2,5-DHBA from aspirin/salicylic acid, sufficient to cause inhibition of cell proliferation, allowing aspirin to act locally on colorectal tissues, thus adding merit to our hypothesis. In our opinion, locally generated metabolites will provide a more direct effect on colorectal tissues against CRC as compared to the indirect mechanism involving sequential steps proposed in platelet hypothesis through COX-1 and COX-2 inhibition. *In vivo* studies utilizing germ-free mice should shed light on the role of aspirin/salicylic metabolites and the microflora in aspirin's chemo-preventive properties.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

GJB and RS conceptualized the study. CKV, RD and TL contributed to the experimental work. DRK and RS conducted the formal analysis (molecular docking). GJB and RS performed the experimental investigation and analysis. RS and GJB wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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