The aspirin metabolite, salicylate, inhibits 7,12-dimethylbenz[a]anthracene-DNA adduct formation in breast cancer cells

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Abstract. There is evidence that aspirin and other non-steroidal anti-inflammatory drugs may be protective agents against cancer in the gastrointestinal tract. These effects are particularly well documented for the colon and rectum. Some epidemiological and experimental studies have suggested that aspirin could also be a chemopreventive agent against breast cancer. We investigated the effects of the aspirin metabolite, salicylate (SA), on 7,12-dimethylbenz[a]anthracene (DMBA)-DNA adduct formation as well as on the expression of the enzymes involved in the carcinogen bioactivation pathway, in particular cytochrome P450 1A (CYP1A) and cyclooxygenases (COX-1 and COX-2). The effects of the test drug were examined in both the human mammary carcinoma cell line, MCF-7, and mammary cells derived from DMBA-induced rat mammary tumours (RMTCs). In this study, we also reported the effects of SA on cell growth and viability in breast cancer cells (BCCs). The results demonstrated that DMBA-DNA adduct formation in both cancer cell lines was inhibited by SA at concentrations of ≥ 2.5 mM. CYP1A was undetectable in RMTCs while CYP1A induction by β-naphthoflavone in MCF-7 cells was significantly inhibited by SA in a concentration-dependent manner. Aspirin did not affect COX-1 expression in either of the BCCs. COX-2 was not detected in MCF-7 cells, but its expression in RMTCs was inhibited by SA treatment, which also significantly reduced BCC growth, but failed to cause cell death by necrosis or apoptosis. These data suggest that inhibition of DMBA-DNA adduct formation may contribute to aspirin breast cancer chemopreventive action and indicate that this drug can act in the first stage of carcinogenesis.

Introduction

The high prevalence of breast cancer provides a strong rationale for identifying new compounds, both natural and synthetic, for use as cancer chemopreventive agents. There is evidence that a variety of chemicals can inhibit, delay, and/or reverse cancer induced by either environmental insults and/or lifestyle. Non-steroidal anti-inflammatory drugs (NSAIDs) are among the molecules that have emerged as promising candidates for breast cancer prevention (1). Their chemopreventive action in breast cancer has been suggested in several epidemiological (2-11) and experimental studies (12-18). Different mechanisms have been proposed to explain the antitumourigenic action of NSAIDs, including inhibition of cell proliferation and angiogenesis, stimulation and promotion of apoptosis, and inhibition of cyclooxygenase-2 (COX-2)-mediated inflammation, immunosuppression and enhanced invasiveness (19-31). Many chemical agents with antitumourigenic properties can modify the mutagenic and carcinogenic effects of environmental carcinogens such as 7,12-dimethylbenz[a]anthracene (DMBA). For the initiation stage of carcinogen-induced tumourigenesis, a metabolic activation of the carcinogen to reactive metabolites that bind covalently to DNA is required (32). In particular, DMBA is biotransformed by cytochrome P450s (CYPs) to electrophilic dihydrodiolepoxides leading to DNA modifications, mutagenicity, and carcinogenicity. This may proceed through a primary metabolic step carried out by hepatic metabolism, or through complete metabolic activation in the breast in situ, or a combination of both processes (33). It has been reported that many xenobiotic metabolizing enzymes are expressed in the breast and that cyclooxygenase (COX), also referred to as prostaglandin H-synthase (PGHS), may play an important role in carcinogen bioactivation in this or other extrahaepatic tissues (34). A variety of chemopreventive agents have been shown to inhibit the in vivo formation of DMBA-induced DNA adducts in rodent mammary cells (35-44).

The main objective of this study was to investigate the effects of the aspirin metabolite, salicylate (SA), on DMBA-DNA adduct formation as well as on the expression of the enzymes involved in the carcinogen bioactivation pathway, in particular CYPs and COX. For this purpose we used the human breast cancer cell line, MCF-7, because it derives from
the mammary epithelium, the target tissue of DMBA, and because carcinogen activation has been well characterized in these cells (45,46). Furthermore, recent studies have demonstrated that MCF-7 cells are similar to normal human mammary epithelial cells with regard to expression and activity of CYP1A1, the principal CYP isoform involved in DMBA bioactivation in the liver (47-49). Since a recent in vivo study has shown that SA inhibited DMBA-induced rat mammary carcinogenesis (50), the same goal was pursued in mammary cells derived from DMBA-induced rat mammary carcinomas (RMTCs). In this work, we also reported the effects of SA on cell growth and viability in breast cancer cells (BCCs).

Materials and methods

DMBA tumour induction, mammary tumour cell separation and culture. Mammary tumours were induced in 30-day-old outbred female Sprague-Dawley rats (Charles River, Calco, Lecco, Italy) by three intragastric intubation of 1 ml DMBA (10 mg/ml in olive oil). All animal procedures were carried out in compliance with the EC Directive 86/609/EEC and with the Italian law regulating experiments on animals. When the tumour mean diameter was approximately 1.5 cm, animals were sacrificed by carotid bleeding under CO2 anaesthesia and tumour tissue was isolated under aseptic conditions to obtain primary cultures of RMTCs (51,52). Tissue was freed from necrotic areas and minced with a scalpel in RPMI-1640 medium supplemented with 5% antibiotics. The minced tissue was resuspended in the same medium containing 0.3% EDTA, and 20% glycerol and sonicated in order to obtain a homogenous membrane suspension. Protein concentration expression and induction by Western blot analysis

Sample preparation. CYP1A: to induce CYP1A expression, cells were incubated for 24 h in the presence of medium containing 0.1% dimethyl sulfoxide (DMSO) as vehicle or in the presence of 10 μM β-naphthoflavone (β-NF) or 10 μM DMBA, dissolved in DMSO, alone or in association with SA 2.5, 3.0 and 5.0 mM. Afterwards, microsomes were isolated as follows: detached cells were washed with PBS and homogenized by sonication at 4°C in 20 mM Tris-HCl, 1.15% KCl, 1.0 mM EDTA, 1.0 mM phenylmethylsulfonyl fluoride (PMSF), 2.0 μg/ml aprotinin, and 2.0 μg/ml leupeptin. The homogenates were centrifuged for 30 min at 10,000 x g at 4°C and supernatants were subjected to further centrifugation at 100,000 x g for 60 min at 4°C. The resulting microsomal pellets were resuspended in 10 mM Tris-acetate, 1.0 mM EDTA, and 20% glycerol and sonicated in order to obtain a homogenous membrane suspension. Protein concentration was determined for each sample using the Lowry assay (55).

COX-1: cells were exposed for 24 h to 0.1% DMSO as vehicle, and 1 μM DMBA dissolved in DMSO, alone or in association with SA 2.0, 2.5, 3.0 or 5.0 mM. After trypsinization and washing in cold PBS, whole-cell lysates were prepared by incubating cells with lysis buffer (50 mM HEPES, 250 mM NaCl, 10% glycerol, 1.0% Triton X-100, 1.5 mM MgCl2, 1.0 mM PMSF, 1.0 mM EGTA, 2.0 mM
Na<sub>2</sub>VO<sub>4</sub>, 10 μg/μl aprotinin and 10 μg/μl leupeptin] on ice for 1 h as described by Novelli et al (59). Results were expressed as average of cpm taken up by each group of treatment.

Measurement of cell viability. Cell viability was assessed using the following methods: propidium iodide (PI, Sigma-Aldrich) staining assay, lactate dehydrogenase (LDH, Sigma-Aldrich) release, Hoechst-33342 (Sigma-Aldrich) staining assay and DNA analysis.

The PI staining assay is based on necrosis-related cell membrane permeation to IP. Cells were seeded at 30,000 cells/well on 24-well plates. After 48 h of culture, cells were washed with PBS and incubated for 24 h in 1.0% FBS-containing medium in the presence of SA at concentrations of 0, 2.0, 2.5, 3.0 and 5.0 mM. Next, the samples were incubated for 15 min at room temperature in the dark with 1 μg/100,000 cells PI. After incubation, adherent cells were detached using trypsin (0.5% trypsin/0.1% EDTA in PBS). Detached cells were harvested by centrifugation at 80 x g for 10 min and resuspended to 0.2x10<sup>6</sup> cells/ml in serum-free medium. Cells were applied to glass slides (5x10<sup>4</sup> cells/slide) by cyt centrifugation at 20 x g for 5 min (Cytospin cytocentrifuge, Shandon, Inc., Pittsburg, PA). After fixing with ethanol/diethyl ether 1:1 for 10 min at room temperature, all slides were sealed with glycerol and analyzed using a fluorescent microscope Leica PMIRE-2 with a UV filter and a x40 oil-immersion lens (Leica Microsystems Wetzlar GmbH, Wetzlar, Germany).

The leakage of LDH into the culture medium after exposure of cells to the different concentrations of SA for 24 h was measured using a colorimetric method (60). Briefly, 0.1 ml samples were mixed with 0.1 ml of LDB substrate mixture (5.4x10<sup>-2</sup> M L(+)- lactate, 6.6x10<sup>-4</sup> M 2-[4-iodophenyl]-3-[4-nitrophenyl]-5 phenyl tetrazolium chloride, 2.8x10<sup>-4</sup> M phenazine methosulphate, 1.3x10<sup>-3</sup> M β-nicotinamide adenine dinucleotide 1:1 for 10 min at room temperature, with a run-time of 10 min and reading intervals of 5 sec. The total release was determined by sonication untreated cells. The spontaneous release was determined in supernatants from untreated cells. Results were expressed as the percentage of total release.

The Hoechst-33342 staining assay was utilized in order to evaluate changes in cell nuclear morphology after exposure to SA. Cells were cultured, treated and applied to glass slides as described for IP staining. After fixing with ethanol/diethyl ether 1:1 for 10 min at room temperature, the cells were washed three times with 0.1 M PBS and sealed with glycerol.

Nuclear morphology was examined using a Leica fluorescence microscope Leica PMIRE-2 as described for the IP test. Nuclei were considered to have the normal phenotype when glowing bright and homogeneously. Apoptotic nuclei can be identified by condensed chromatin gathering at the periphery of the nuclear membrane or a total fragmented morphology of nuclear bodies.

To evaluate SA cell toxicity, the DNA fragmentation assay was also performed. In brief, cells, cultured and treated as previously described were incubated in 30 μl/1x10<sup>6</sup> cell
Lysis buffer containing 10 mM EDTA, 0.5% SDS and 0.5 mg/ml proteinase K in 50 mM Tris-HCl (pH 8.0) for 1 h at 50˚C. The reaction was stopped in ice and each sample was incubated with 10 μg/ml RNAase for 1 h at 50˚C. The samples were further incubated for 10 min at 70˚C to inactivate both proteinase K and RNAase. After addition of 0.3 M ammonium acetate, DNA was extracted using cold ethanol, pelleted and resuspended in sterile water. The amount and the purity of DNA were determined by spectrophotometry. The degree of fragmentation was analyzed using 2% agarose gel electrophoresis and ethidium bromide staining (61).

Statistical analyses. Data were expressed as means ± SD and subjected to one-way ANOVA followed by the Newman-Keuls test to determine the statistical significance of the differences between the experimental groups. A p<0.05 was considered significant.

Results

Effects of SA on DMBA-DNA adduct formation. The effect of SA on DMBA-DNA adduct formation in BCCs was examined in control cultures. Exposure to 0.1 μg/ml [3H]DMBA for 24 h resulted in the formation of 1974±381 and 2040±365 fmoles adducts per mg DNA in MCF-7 cells and in RMTCs, respectively. Exposure of either cell type to [3H]DMBA in the presence of different concentrations of SA significantly reduced DMBA-induced DNA adduct formation at concentrations ≥2.5 mM (Tables I and II).

### Table I. Effect of salicylate on [3H]DMBA-DNA adduct formation in MCF-7 cells.

<table>
<thead>
<tr>
<th>Group</th>
<th>fmoles [3H-DMBA/mgDNA] (mean ± SD)</th>
<th>Statistics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1974±381</td>
<td>-</td>
</tr>
<tr>
<td>SA 2.0 mM</td>
<td>1671±353</td>
<td>ns</td>
</tr>
<tr>
<td>SA 2.5 mM</td>
<td>1330±320</td>
<td>b</td>
</tr>
<tr>
<td>SA 3.0 mM</td>
<td>709±340</td>
<td>b</td>
</tr>
<tr>
<td>SA 5.0 mM</td>
<td>639±389</td>
<td>b</td>
</tr>
</tbody>
</table>

*a Three independent experiments each with n=3; ns: not significant.

### Table II. Effect of salicylate on [3H]DMBA-DNA adduct formation in RMTCs.

<table>
<thead>
<tr>
<th>Group</th>
<th>fmoles [3H-DMBA/mgDNA] (mean ± SD)</th>
<th>Statistics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2040±365</td>
<td>-</td>
</tr>
<tr>
<td>SA 2.0 mM</td>
<td>1814±212</td>
<td>ns</td>
</tr>
<tr>
<td>SA 2.5 mM</td>
<td>1391±150</td>
<td>b</td>
</tr>
<tr>
<td>SA 3.0 mM</td>
<td>551±130</td>
<td>b</td>
</tr>
<tr>
<td>SA 5.0 mM</td>
<td>533±147</td>
<td>b</td>
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*a Three independent experiments each with n=2. ns, not significant.

bp<0.05 vs control.

Figure 1. Effects of DMBA or ß-NF alone or in association with different concentrations of SA on CYP 1A1/1A2 expression in microsomes from MCF-7 cells: A, vehicle; B, DMBA, 10 μM; C, ß-NF 10 μM; D, ß-NF 10 μM + SA 2.5 mM; E, ß-NF 10 μM + SA 3.0 mM; F, ß-NF 10 μM + SA 5.0 mM; G, positive control (rat liver microsomes induced with ß-NF). The immunoblots shown in the figure are from one representative experiment out of three. CYP1A1/1A2 levels are expressed as arbitrary units obtained from densitometric scanning analysis of the immunoreactive bands. The same membrane was reprobed with an anti-ß-actin antibody to verify equalization of protein loading in the different lanes. *p<0.05 vs C (ß-NF).

Figure 2. Effects of DMBA alone or in association with different concentrations of SA on COX-1 expression in MCF-7 cells lysate samples: A, vehicle; B, DMBA 1 μM; C, DMBA 1 μM + SA 2.0 mM; D, DMBA 1 μM + SA 2.5 mM; E, DMBA 1 μM + SA 3.0 mM; F, DMBA 1 μM + SA 5.0 mM. The immunoblots shown in the figure are from one representative experiment out of three. COX-1 levels are expressed as arbitrary units obtained from densitometric scanning analysis of the immunoreactive bands. The same membrane was reprobed with an anti-ß-actin antibody to verify equalization of protein loading in the different lanes.

*p<0.05 vs C (ß-NF).
SA-mediated modulation of CYP1A, COX-1 and COX-2. CYP1A: immunoblots carried out in microsomal fractions with the appropriate antibody to assess the constitutive expression and induction of CYP1A in each cell type showed a very low protein expression in untreated MCF-7 cells. Following treatment with 10 μM DMBA, there was a slight and not significant increase in CYP1A1/1A2 immunoreactive protein. In contrast, protein expression was more notably enhanced (+17%) in microsomes from 10 μM ß-NF treated cells; this phenomenon was significantly inhibited by SA in a concentration-dependent manner (Fig. 1). CYP1A1/1A2 was seemingly neither constitutively expressed nor induced in RMTCs under the same conditions (data not shown).

COX-1 and COX-2: immunoblotting results (Figs. 2 and 3) indicated that blots for COX-1 protein from MCF-7 cells and RMTCs were similar. COX-1 expression was little affected by DMBA alone and the concurrent exposure to SA did not result in appreciable changes in the enzyme expression, irrespective of the cell source. COX-2 was undetectable in MCF-7 cells (data not shown), but clearly detectable in RMTCs. COX-2 expression was seemingly not modified by DMBA alone, while cells exposed to both DMBA and SA displayed a significant decrease in protein signal (Fig. 4).

Effects of SA on breast cancer proliferation. MCF-7 cells and RMTCs were incubated for 24 h with SA at concentrations
ranging from 2.0 to 5.0 mM. As revealed by the MTT assay, SA significantly decreased the number of both types of BCC at concentrations of ≥2.0 mM (Figs. 5 and 6). [3H]-thymidine incorporation studies showed that, after 24-h incubation, the inhibition of DNA replication by SA was significant at concentrations of ≥2.5 mM (Figs. 7 and 8).

Effects of SA on breast cancer cell viability. The evaluation of SA cell toxicity was performed using different assays to discriminate between necrosis and apoptosis. As assessed by the PI assay (Fig. 9), neither of the BCCs exhibited any morphological change after the exposure for 24 h to SA at the lowest (2.0 mM) or highest (5.0 mM) tested concentrations. Likewise, cell damage evaluated in terms of LDH release was not induced by SA treatment (Fig. 10). We used Hoechst 33342 staining and DNA ladder assay to study the effects of SA on apoptosis. No apoptotic bodies containing nuclear fragments were found in BCCs exposed to SA 2.0 or 5.0 mM for 24 h (Fig. 11). Gel electrophoresis of DNA from BCCs treated with increasing concentrations of SA for 24 h revealed a 'ladder' pattern that indicated no DNA degradation (Figs. 12 and 13).

Discussion
The present study shows that treatment of mammary cancer cells for 24 h with different concentrations of SA inhibits...
DMBA-DNA adduct formation, a phenomenon which is generally accepted as a critical step in the mechanism by which polycyclic aromatic hydrocarbons (PAHs) cause mutations leading to the induction of cancer in the target organs (62). The exposure of cells to 2.5 mM SA results in a significant inhibition of DMBA-DNA adduct formation that reaches a maximum value at 3.0 mM, a concentration compatible with drug plasma levels measurable during treatment of chronic inflammatory diseases (63,64). This effect may contribute to the aspirin breast cancer chemopreventive action observed in clinical trials and epidemiological and experimental studies; it also indicates that this drug can act in the first stage of carcinogenesis. As mentioned above, DMBA, in common

Figure 10. Effect of different concentrations of SA on lactate dehydrogenase (LDH) release of MCF-7 cells and RMTCs. Results are expressed as percentage of total release.

Figure 11. Morphological features of Hoechst 33342-stained BCCs either untreated (a and d) or exposed for 24 h to SA 2 mM (b and e) or 5 mM (c and f). Hoechst 33342-staining was used to visualize chromatin condensation or fragmentation, a typical phenomenon of cells undergoing apoptosis. Fluorescent microphotographs of Hoechst 33342-stained BCCs indicate no apoptotic nuclei (condensed or fragmented) after exposure to SA.

Figure 12. Analysis of DNA fragmentation by agarose gel electrophoresis after treatment of MCF-7 cells with different concentrations of SA: A, no treatment; B, SA 2.0 mM; C, SA 2.5 mM; D, SA 3.0 mM; E, SA 5.0 mM; F 1 kb DNA marker. The result presented is typical of 3 separate experiments.

Figure 13. Analysis of DNA fragmentation by agarose gel electrophoresis after treatment of RMTC cells with different concentrations of SA: A, no treatment; B, SA 2.0 mM; C, SA 2.5 mM; D, SA 3.0 mM; E, SA 5.0 mM; F 1 kb DNA marker. The result presented is typical of 3 separate experiments.
with other PAHs, requires metabolic activation by CYP-dependent monoxygenases, mainly CYP1A1 isoforms, or other oxidases before it can induce malignant transformation. As reported in the literature, prior exposure of MCF-7 cells to CYP inducers, such as PAHs or β-NF, enhances CYP1A1 expression (47-49). In our experiments, CYP1A was markedly induced by β-NF and less by DMBA. Since both chemicals share a common induction mechanism, we evaluated the effects of SA on CYP1A1/1A2 expression in cells exposed to β-NF. As detected by Western blotting, microsomes from β-NF-treated MCF-7 cells showed a significant decrease in this protein after SA exposure. In contrast, we did not observe constitutive or inducible CYP1A1/1A2 protein in RMTCs. Both the expression and induction of this enzyme have been well documented in rat mammary glands (65) but, to the best of our knowledge, there is no study on CYP1A1 expression in DMBA-induced mammary tumours. Indeed, there are extensive studies on CYP expression in chemically-induced rat/mouse liver tumours revealing a decrease of the expression of this enzyme in hepatic tumours compared with adjacent non-neoplastic tissue (66-68). As reported by Williams and Phillips (33), COX may play an important role in carcinogen bioactivation in extrahepatic tissue, especially where CYP-mediated enzyme activity is low. Western blot analysis revealed the presence of COX-1 in both cell types. In line with the results of Din et al (69), COX-2 was not detectable in MCF-7 cells, but was present in RMTCs in agreement with Ghezzo et al (50), who reported COX-2 expression in DMBA-induced rat mammary tumours. Prior exposure of cells to DMBA did not affect COX expression. There have been no studies reporting a direct induction of COX expression after exposure of cells or animal tissue to DMBA. Jang et al (12) investigated the expression of COX-1 and COX-2 in normal rat mammary glandular epithelium and in the various stages of DMBA-induced rat mammary carcinogenesis. They observed negligible COX-1 expression and no COX-2 expression in normal mammary glands; DMBA treatment, in itself, did not modify COX expression. On the contrary, DMBA-induced mammary gland neoplastic transformation enhanced COX-1 expression and induced COX-2 expression in a high percentage of tumours. Data from the present study indicate that SA addition did not affect COX-1 expression in BCCs but inhibited COX-2 expression in RMTCs. Until recently, there were no studies examining the effect of aspirin on COX-1 gene expression in human breast cancer or rodent mammary tumours, although Robertson et al (70) reported that ibuprofen was able to inhibit COX-1 and COX-2 gene expression in DMBA-induced rat mammary tumours. Even so, we can assume that SA inhibits COX-1 activity in BCCs by a well-known mechanism, i.e. rapid reversible binding followed by covalent modification (acylation) of Ser 530 of COX-1 (71). Although the exact mechanism by which SA inhibits DMBA-DNA adduct formation in BCCs remains to be established, according to our results this effect may be due to SA’s ability to decrease the expression of the enzymes involved in carcinogen bioactivation.

Since several reports suggest inhibition of tumour cell growth and induction of apoptosis as two possible explanations for the anti-tumour effects of NSAIDs (72), we have also investigated the effects of SA on BCC proliferation and viability in vitro. In line with the results of previous investigations (21,28,73), SA proved effective in the inhibition of BCC growth but failed to cause cell death by necrosis or apoptosis. The latter finding is in contrast with previous reports on NSAID-induced apoptosis in breast cancer cells but agrees with the results of Din et al (69), who showed that aspirin had no effect on viability and apoptosis in breast cancer cell lines. These differences may be explained by considering that induction of apoptosis was observed in studies using NSAIDs other than aspirin (20,23,74,75), while SA-induced apoptosis was observed only after long exposures (48-96 h) to high concentrations of drug beyond the therapeutic range (73).

In conclusion, the present study demonstrates that SA significantly inhibits cell proliferation and DMBA-DNA adduct formation in the two tested types of BCC at concentrations which do not affect cell viability. The latter effect is possibly mediated via modulation of the expression of the enzymes involved in carcinogen bioactivation (CYP1A1/1A2 and COX), even if it is not possible to exclude other mechanisms, such as the induction of phase II detoxifying enzymes. Inhibition of adduct formation by SA represents a further mechanism responsible for aspirin chemopreventive efficacy that can be added to the several antitumour actions previously documented for NSAIDs. In our opinion, the antitumour effect exerted by SA and related agents is probably due to different actions of the drugs, strictly correlated in many cases, that collectively contribute to the final effect.

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References


