

## Differential Growth Inhibition by the Aspirin Metabolite Salicylate in Human Colorectal Tumor Cell Lines: Enhanced Apoptosis in Carcinoma and *In Vitro*-transformed Adenoma Relative to Adenoma Cell Lines<sup>1</sup>

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### Abstract

Regular aspirin intake may reduce the risk of colorectal cancer by 50%. However, the mechanism of this chemopreventive effect is not known. The effect of the aspirin metabolite salicylate on the growth of human colorectal tumor cell lines was determined. Salicylate showed dose-dependent inhibitory effects on all of the cell lines (IC<sub>50</sub>, 1.65 ± 0.36 to 7.38 ± 1.08 mM), yet carcinoma and *in vitro*-transformed adenoma cell lines were more sensitive than adenoma cell lines. Salicylate caused all cell lines to accumulate in G<sub>0</sub>-G<sub>1</sub> and induced apoptosis in carcinoma and *in vitro*-transformed adenoma cell lines but not in all adenoma cell lines. In those adenoma lines that did show salicylate-induced apoptosis, the extent was considerably less than that in the more transformed cell lines. The ability of salicylate to induce cell cycle arrest and apoptosis and, in particular, the increased sensitivity of cells at later stages of neoplastic progression may be mechanistically important in the chemopreventive action of aspirin toward colorectal cancer.

### Introduction

Colorectal cancer remains one of the most common malignancies in the westernized world, and current treatment strategies have little effect on survival (1). However, NSAIDs<sup>3</sup> such as aspirin and sulindac have recently shown considerable promise as agents effective in the chemoprevention of colorectal cancer. Data relating the effects of NSAIDs to human cancer are derived largely from epidemiological studies of aspirin use and the risk of colorectal cancer and clinical trials of sulindac for colonic polyposis. The epidemiological studies (reviewed in Refs. 1 and 2) indicate an approximate 50% reduction in the incidence of, or death from, colorectal cancer among regular users of aspirin. Results from clinical trials broadly support the chemopreventive action of NSAIDs apparent in the epidemiological studies. For example, sulindac causes regression of colorectal polyps in familial adenomatous polyposis (1, 2). Additionally, NSAIDs also have chemopreventive effects in rodent models of colorectal carcinogenesis, reducing the incidence and multiplicity of premalignant and malignant lesions (2, 3). Although these studies indicate a link between NSAID use and a reduced risk of colorectal cancer, the mechanism of chemoprevention remains unknown. It is probable that it results, at least in part, from the inhibitory effects of NSAIDs on prostaglandin biosynthesis (4). In this respect, it is notable that recent studies have shown prostaglandin E<sub>2</sub> levels to be progressively increased from that in control mucosa to adenomatous polyps and then to adenocarcinomas (5). A reduction in prostaglandin levels resulting from NSAID treatment may have a number of potentially chemopreventive

effects, reflecting the diversity of prostaglandin action in the intestine. For example, it may result in a decrease of mitogenic stimulation, restoration of the immune response, and a reduction in the formation of potential carcinogens (4, 6). However, two recent reports of studies using a single colorectal carcinoma cell line (7, 8) have suggested apoptosis as another mechanism for the chemopreventive effects of sulindac and its derivatives, which significantly does not necessarily involve the inhibition of prostaglandin biosynthesis (7). Although aspirin is the only NSAID to have been investigated widely in epidemiological studies and is substantially deacetylated to salicylate on the first pass through the portal circulation (9), there are no studies of the effects of salicylate on colonic epithelial cells. Also, it is likely that the relative sensitivity of cells at different stages of tumor progression is critical to the chemopreventive properties of NSAIDs, yet there are no reports of the effects of NSAIDs on premalignant colonic epithelial cells. Consequently, we chose to investigate the effect of salicylate on the proliferation and apoptosis of a range of colorectal tumor cell lines, including those representing early stages of tumor progression. The results of this study provide a new and significant insight into the effects of salicylate on human colorectal adenoma and carcinoma cell lines and a basis for the chemopreventive effect of aspirin.

### Materials and Methods

**Cell Lines and Culture Conditions.** The human colorectal tumor cell lines used in this study are detailed in Table 1 (10–16). In addition to tumorigenic carcinoma cell lines, they include non-tumorigenic adenoma cell lines and derivatives of these that have been transformed *in vitro* either to tumorigenicity or to anchorage independence. Unless otherwise stated, they were grown on tissue culture plastic in DMEM (Life Technologies, Inc.) with 20% fetal bovine serum (batch selected) as described previously (11), except that the DMEM was not supplemented with hydrocortisone sodium succinate. The inclusion of hydrocortisone may have interfered with the mobilization of arachidonic acid. This is the substrate for cyclooxygenase, both isoforms of which are inhibited by NSAIDs. The non-trypsinizable adenoma cell line PC/AA was routinely maintained in the presence of Swiss 3T3 feeder cells (10), but was grown on plastic in the absence of feeder cells for these studies.

**Treatment with Sodium Salicylate.** Sodium salicylate was prepared as a 50 mM stock solution in water (tissue culture tested; Life Technologies, Inc.) with the final pH adjusted to 7.2. Water alone was added to control cultures. Except in the case of the PC/AA line, exponentially growing cells were trypsinized prior to seeding at a density so that there were 2–3 × 10<sup>6</sup> cells/T25 flask at the time of treatment (3–5 days after seeding). The non-trypsinizable PC/AA adenoma cells were passaged using dispase (10) and seeded for experiment in DMEM in the absence of 3T3 feeder cells; again, at a density so that there were 2–3 × 10<sup>6</sup> cells/T25 flask at the time of treatment. Cells were treated in triplicate with 1–5 mM sodium salicylate (Sigma, Poole, United Kingdom) for 4 days, and then the attached cells (those remaining adhered to the tissue culture flask) and floating cells (those having detached from the tissue culture flask) were counted separately and stained with acridine orange for analysis by fluorescence microscopy (see below). IC<sub>50</sub> values were determined from dose-response curves of the salicylate concentration *versus* attached cell yield.

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<sup>3</sup> The abbreviation used is: NSAID, nonsteroidal anti-inflammatory drug.

Table 1 Human colorectal tumor cell lines used

Cell line <sup>a</sup> (passage no.)	Description	Reference
<b>Adenoma cell lines<sup>b</sup></b>		
1:PC/AA (18–20)	Adenoma derived from familial adenomatous polyposis patient, nonclonogenic	10
2:AA/C1 (75–82)	Clonogenic (passaged with trypsin) variant of PC/AA	11
3:RR/C1 (32–38)	Sporadic adenoma-derived, clonogenic	12
4:RG/C2 (44–49)	Sporadic adenoma-derived, clonogenic	13
<b><i>In vitro</i> transformants of adenoma cell lines</b>		
5:AA/C1/SB10 (173–181)	Chemical carcinogen transformed variant of AA/C1, tumorigenic	11
6:RG/C2/GV (127–134)	Chemical carcinogen and radiation transformed variant of RG/C2 Anchorage independent but nontumorigenic	14 <sup>c</sup>
<b>Carcinoma cell lines<sup>b</sup></b>		
7:HT29 (284–290)	Sporadic carcinoma	15
8:S/KS (42–48)	Sporadic carcinoma	16

<sup>a</sup> The numbering of the cell lines corresponds to that used in the figures and text.

<sup>b</sup> The adenoma cell lines (lines 1–4) are nontumorigenic in athymic nude mice. The carcinoma cell lines (lines 7 and 8) are tumorigenic in athymic nude mice.

<sup>c</sup> RG/C2/GV was derived from RG/C2 by treatment with 1  $\mu$ g/ml *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, selection for growth in soft agar followed by irradiation with five fractions of 1.5 Gy each at 6-week intervals.

**Measurement of Apoptosis.** As we and others have described previously, the level of apoptosis in cultured epithelial cell lines can be assessed by measuring the proportion of the total cell population that has detached from the cell monolayer and is floating in the medium and determining the fraction of these floating cells that are apoptotic (16–18). Following salicylate treatment, the proportion of the total cell population that was floating was determined for each salicylate dose. To determine whether the induction of floating cells was due to apoptosis, the proportion of the attached and floating cell populations that were apoptotic was established by staining with acridine orange (5  $\mu$ g/ml in PBS) and analysis by fluorescence microscopy as described previously (16, 19). Apoptotic cells were identified by their characteristically condensed chromatin stained by the acridine orange. Necrotic cells were identified as cells with poorly staining “hollow” nuclei. Acridine orange-stained cells were photographed using Fujichrome Provia (ISO 400) film. Statistical analysis was carried out using Student's unpaired *t* test; *P* < 0.05 was considered to be statistically significant.

**Analysis of Cell Cycle Distribution.** For flow cytometric analysis, cells were treated with 5 mM salicylate for 24 to 72 h. Samples of attached cells were fixed with 70% ethanol and stained with 20  $\mu$ g/ml propidium iodide (Sigma) before being analyzed for red (FL2) fluorescence on a FACScan (Becton Dickinson), as detailed previously (19). The cell cycle distribution was calculated from the resultant DNA histograms using Cell FIT software, based on a rectangular S-phase model.

## Results

**Salicylate Differentially Inhibits the Growth of Colorectal Adenoma and Carcinoma Cell Lines.** The effect of salicylate on the growth of colorectal adenoma, *in vitro*-transformed adenoma, and carcinoma cell lines (Table 1) was determined after 4 days of treatment. In the range of 1–5 mM, salicylate showed dose-dependent inhibitory effects on the attached cell yield of all cell lines tested (Fig. 1, A–C). The dose-response curves of each cell line were used to determine the specific IC<sub>50</sub> value for the reduction of cell numbers by salicylate (Fig. 1D). There was considerable variation in the sensitivities of the cell lines to the growth inhibitory effects of salicylate with IC<sub>50</sub> values ( $\pm$ SD) ranging from 1.65  $\pm$  0.36 to 7.38  $\pm$  1.08 mM (Fig. 1D). However, in all cases the adenoma cell lines were less sensitive than the carcinoma and *in vitro*-transformed adenoma cell lines to the growth inhibitory effects of salicylate. Only in a comparison of the IC<sub>50</sub> value of the RG/C2 adenoma line and that of the HT29 carcinoma line was this difference found not to be significant (*P* = 0.1079; Fig. 1D, lines 4 and 7). The differential effect was apparent not only between unrelated cell lines at different stages of neoplastic transformation but also between adenoma lines and their *in vitro*-transformed derivatives. Progression of the parental PC/AA adenoma cell line (the least sensitive of the cell lines to salicylate) through the clonogenic variant AA/C1 and to the tumorigenic line AA/C1/SB10 (Table 1; Ref. 11) resulted in the reduction of the IC<sub>50</sub> value for salicylate from 7.38  $\pm$  1.08 mM to 4.69  $\pm$  0.45 mM to 2.77  $\pm$  0.56 mM, respectively (Fig.

1D, lines 1, 2, and 5). Thus, conversion of a non-tumorigenic adenoma cell line to a tumorigenic adenocarcinoma resulted in an increased sensitivity to the growth inhibitory properties of salicylate. Similarly, progression of the RG/C2 adenoma cell line (IC<sub>50</sub>, 3.89  $\pm$  0.15 mM) to an anchorage-independent line with increased colony-forming efficiency on plastic was associated with a reduction of the IC<sub>50</sub> value for salicylate to 2.57  $\pm$  0.22 mM (Fig. 1D, lines 4 and 6).

To examine a possible mechanism by which salicylate had an antiproliferative effect, the cell cycle phase distribution of cell lines 1–8 (Table 1) was compared for control and 5 mM salicylate-treated cultures (data not shown). In all of the cell lines tested, salicylate treatment caused an accumulation of cells in G<sub>0</sub>-G<sub>1</sub> and a decrease in the proportion of cells in the S-phase. Notably, the cell lines that were more sensitive to the growth inhibitory properties of salicylate (as determined by the IC<sub>50</sub> values) did not necessarily exhibit an increased cell cycle arrest when compared with less sensitive cell lines.

**Salicylate Induces Apoptosis in Colorectal Carcinoma and *in Vitro*-transformed Adenoma Cell Lines but Significantly Less So in Adenoma Cell Lines.** Having established that salicylate was growth inhibitory to the colorectal tumor cell lines, we investigated whether salicylate induced apoptosis. We have shown previously that in the routine culture of colorectal epithelial tumor cells, the majority of cells that spontaneously detach from the tissue culture flask and float in the medium are apoptotic (16). Fig. 2 illustrates the effect of salicylate treatment on the proportion of the total cell population (attached and floating) that is floating in the medium. In the carcinoma and transformed adenoma cell lines (lines 5–8; Table 1), salicylate was found to increase, in a dose-dependent manner, the proportion of cells floating. The maximum extent of the induction of cell death in these cell lines ranged from 3.7  $\pm$  0.37-fold to 14.6  $\pm$  1.56-fold the control value. In contrast, adenoma cell lines (lines 1–4; Table 1) were relatively resistant to the induction of cell death by salicylate (Fig. 2). The response was not dose dependent in every case, and the maximum induction of cell death ranged from 1.25  $\pm$  0.13-fold to 1.9  $\pm$  0.09-fold consistency the control value. The maximum induction of cell death was highly significant for the more transformed cell lines (*P* = 0.0003, 0.0015, 0.0005, and 0.0001, respectively, for lines 5–8) but less so for the adenoma AA/C1 (*P* = 0.012) and RR/C1 (*P* = 0.005) cell lines, reflecting the extents of cell death shown in Fig. 2. The induction of cell death was not significant (*P* > 0.05) for the PC/AA and RG/C2 adenoma lines.

To determine whether the increase in floating cells was due to the induction of apoptosis rather than just necrosis, the floating cells were analyzed using fluorescence microscopy following staining with acridine orange. This showed that at the highest salicylate concentration (5 mM) the proportion of floating cells that were apoptotic was equal

to control values (86–95% dependent on cell line). Hence, the increase in the proportion of cells floating in salicylate-treated cultures was due largely to the induction of apoptosis and not simply a result of increased necrosis. Only in the case of S/KS, the most sensitive cell line, was the proportion of floating cells (in 5 mM treated cultures) that were apoptotic slightly reduced compared with control levels (see legend to Fig. 2). At lower doses the apoptotic fraction of the floating

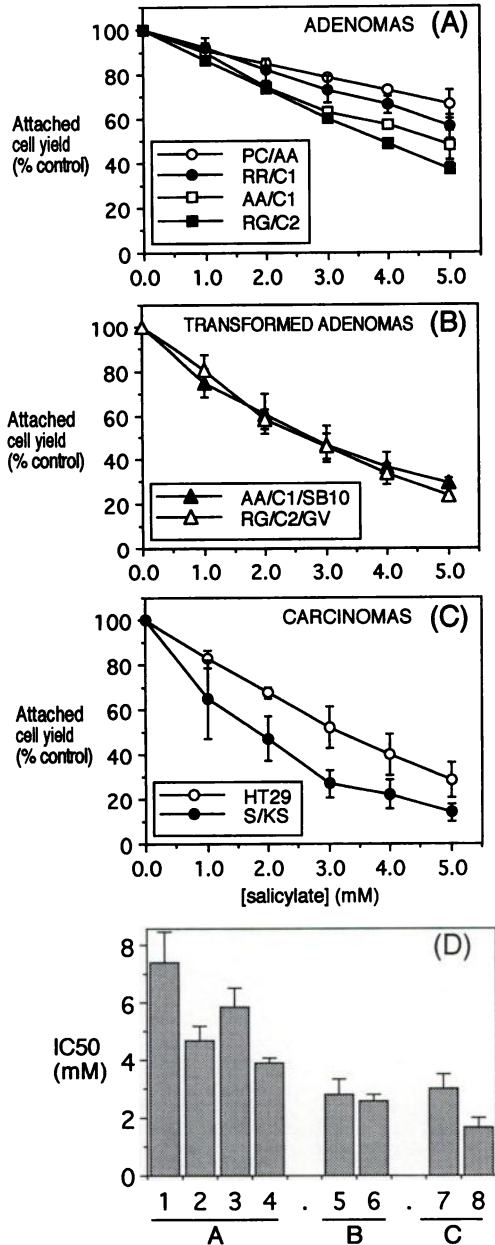


Fig. 1. Dose-dependent growth inhibition of colorectal adenoma (A), *in vitro*-transformed adenoma (B), and carcinoma (C) cell lines after 4 days treatment with the aspirin metabolite salicylate. The attached cell yields (number of cells remaining attached to the tissue culture flask) following salicylate treatment are expressed as percentage of control values. Details of the cell lines are presented in Table 1. Each experiment was carried out with triplicate determinations for each salicylate concentration (0–5 mM). The data presented are the means of the three experiments. Bars, SD. D, differential effect of treatment with the aspirin metabolite salicylate on the proliferation of colorectal tumor cells at different stages of neoplastic progression. The  $IC_{50}$  values are the salicylate concentrations (mM) required to reduce the attached cell yield of each cell line to 50% of that of control levels after 4 days of treatment. They were determined from a line of best fit through each dose-response curve. Those presented are the means of the  $IC_{50}$  values obtained from the three separate dose-response experiments. Bars, SD. Adenomas (A): line 1, PC/AA; line 2, AA/C1; line 3, RR/C1; line 4, RG/C2. *In vitro*-transformed adenomas (B): line 5, AA/C1/SB10; line 6, RG/C2/GV. Carcinomas (C): line 7, HT29; line 8, S/KS.

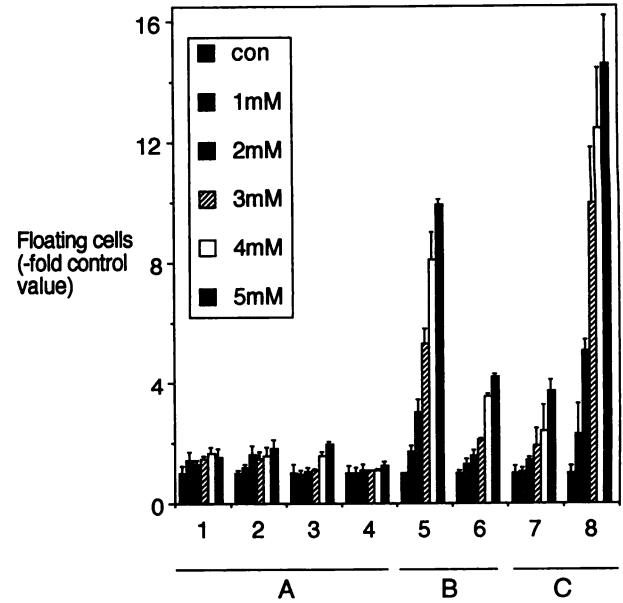


Fig. 2. Dose-dependent induction of apoptosis of colorectal tumor cells *in vitro* following 4 days of treatment with the aspirin metabolite salicylate. The data indicate the level of floating cells, as a proportion of the total cell number (attached and floating), following salicylate (1–5 mM) treatment in relation to the control (spontaneous) level (assigned as 1) for the respective cell line. The control (spontaneous) level (mean percentage  $\pm$  SD) of floating cells for each cell line was as follows: Adenomas (A): 1, PC/AA (2.66  $\pm$  0.67); 2, AA/C1 (0.92  $\pm$  0.11); 3, RR/C1 (6.48  $\pm$  1.81); 4, RG/C2 (6.14  $\pm$  1.61). *In vitro*-transformed adenomas (B): 5, AA/C1/SB10 (1.00  $\pm$  0.02); 6, RG/C2/GV (3.50  $\pm$  0.41). Carcinomas (C): 7, HT29 (4.23  $\pm$  1.05); 8, S/KS (4.87  $\pm$  1.14). The morphology of the (acridine orange-stained) floating cells from control and treated cultures was examined at the end of each experiment to determine whether the increase in floating cells with salicylate treatment was due to apoptosis. For each cell line (apart from S/KS when treated with 5 mM salicylate), the proportion of floating cells that were apoptotic in salicylate-treated cultures was similar to that of control cultures, where this proportion was 86–95% dependent on the cell line. For S/KS (the cell line most sensitive to the growth inhibitory properties of salicylate), the proportion of floating cells that were apoptotic decreased from 94.3%  $\pm$  1.2 in control cultures to 82.5%  $\pm$  2.1 in cultures treated with 5 mM salicylate, with a corresponding increase in cells appearing to be necrotic. Details of the cell lines are presented in Table 1. Each experiment was carried out with triplicate determinations for each salicylate concentration (0–5 mM). The data presented are the means of the three experiments. Bars, SD.

cells was similar to that of the respective control cultures. A representative photomicrograph of apoptotic cells in the floating cell population of control and salicylate-treated cultures of the AA/C1 adenoma cell line is shown in Fig. 3.

## Discussion

Epidemiological and clinical studies have left little doubt that aspirin and related compounds have considerable potential as chemopreventive agents for colorectal cancer. It is therefore important to understand their effect on colorectal tumor cells. This study has shown that the aspirin metabolite salicylate inhibits growth and induces apoptosis in colorectal tumor cells. We have also found salicylate to cause cell cycle arrest in these cells. Recently, it has been shown that sulindac and its derivatives have similar effects on HT29 colon carcinoma cells (7, 8). The response of colorectal carcinoma cells to salicylate, sulindac, and its derivatives may in part explain the chemopreventive nature of these compounds in colorectal cancer. However, a critical issue in cancer chemoprevention or chemotherapy is the relative sensitivity of cells at different stages of neoplastic progression. Thus, the importance of the present study lies not only in the use of the aspirin metabolite salicylate but also of premalignant colorectal tumor cells in addition to carcinoma cells. Colorectal carcinoma and *in vitro*-transformed adenoma cells were found to be more sensitive than colorectal adenoma cells to growth inhibition and, particularly, to the induction of apoptosis by salicylate. Indeed, salic-



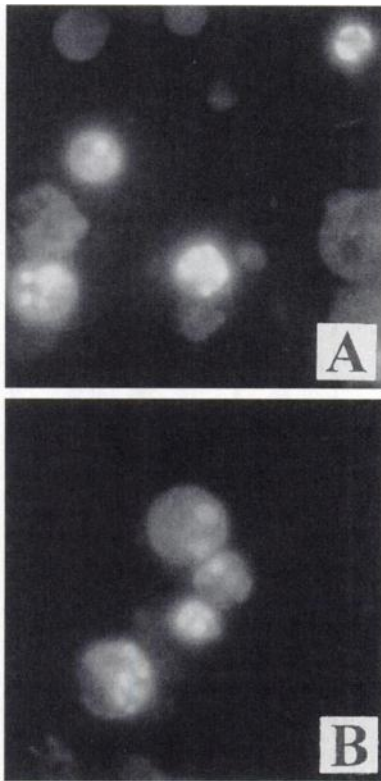


Fig. 3. Morphological features of acridine orange-stained cells from the floating cell population of control (A) and salicylate-treated (B) cultures of the AA/C1 adenoma cell line. The condensed chromatin stained by the acridine orange is typical of apoptotic cells.

ylate failed to induce significant apoptosis in two of the four adenoma cell lines studied. The cell lines that were more sensitive to the growth inhibitory properties of salicylate did not necessarily exhibit an increased cell cycle arrest when compared with less sensitive cell lines. Yet all carcinoma and *in vitro*-transformed adenoma cell lines were dramatically more sensitive than the adenoma cell lines to the induction of apoptosis by salicylate. This suggests that the level of induction of apoptosis by salicylate was the major factor accounting for the differential sensitivities.

Low levels of apoptosis, if not balanced by proliferation, can result in significant tissue regression over time (20). Thus, with prolonged treatment *in vivo*, even minor differences in the sensitivity of cells to salicylate may result in the relative regression of regions of tissue of the more sensitive cell type. The results presented here suggest that these would be the more transformed cells. In this respect, it is notable that sulindac has been shown to cause regression of colorectal polyps in patients with adenomatous polyposis coli (2, 21).

The molecular elements determining the sensitivity of colorectal tumor cells to salicylate are unclear. In the present study, salicylate was found to induce apoptosis in colorectal tumor cells lacking wild-type p53 protein (RG/C2/GV, HT29, and S/KS). This is consistent with evidence suggesting that the induction of apoptosis by sulindac and its derivatives involves a p53-independent mechanism (7, 8). One molecular target for the NSAIDs is cyclooxygenase, a key enzyme in prostaglandin biosynthesis. Two isoforms of cyclooxygenase have been identified, the second of which, COX-2, is expressed in colorectal carcinomas but is rarely detectable in colorectal adenomas or normal colonic tissue (22, 23). A recent study has implicated COX-2 activity in the regulation of apoptosis of rat intestinal epithelial cells (18). It will be of interest to determine, in the cell lines studied here, whether the differential sensitivity to salicylate-induced apoptosis relates to COX-2 expression and prostaglandin biosynthesis. It is possible that other pathways, independent of the inhibition of

prostaglandin biosynthesis, may be involved in the cellular response to NSAIDs. This has been proposed for sulindac sulfone which induces apoptosis in the colon carcinoma cell line HT29 but does not inhibit prostaglandin biosynthesis (7).

Although the molecular mechanisms remain unclear, our study demonstrates cellular responses to salicylate that may contribute to the chemopreventive effects of regular aspirin intake toward colorectal cancer. Of particular importance is the demonstration that *in vitro*-transformed adenoma and carcinoma cell lines are more sensitive than adenoma cell lines to growth inhibition and the induction of apoptosis by salicylate. Such differential effects will be central to the development of NSAID chemoprevention strategies.

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