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ORIGINAL ARTICLE

Yanqun Liu · Kong Weng Eu · Francis Seow-Choen
Peh Yean Cheah

Differential cytostatic effect of sodium salicylate in human colorectal cancers using an individualized histoculture system

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Abstract *Purpose:* Tumor metastasis is the major cause of colorectal cancer (CRC) mortality. Nonsteroidal antiinflammatory drugs (NSAIDs) such as aspirin have been shown to have an antineoplastic effect in CRC cell lines. Different patients, however, exhibit different chemosensitivity. In this study, we assessed the chemotherapeutic potential of sodium salicylate, an aspirin metabolite, by measuring its cytostatic effect in an individualized three-dimensional histoculture system. *Methods:* Histocultured cancer tissues were treated with sodium salicylate at concentrations from 1 to 10 m*M* for 24 or 48 h. Inhibition of DNA synthesis was measured in terms of inhibition of bromodeoxyuridine (BrdU) incorporation. *Results:* The concentration response of individual cancer tissues could be categorized into four groups ranging from the most to the least sensitive to sodium salicylate. Of 20 cancer tissues, 12 (60%) showed a concentration-effect relationship with sodium salicylate in the clinically relevant concentration range (IC_{50} 1.2 ± 0.4 to 3.8 ± 0.5 m*M*). Doubling the exposure time decreased the IC_{50} in four specimens, suggesting that a similar inhibition might be achieved with a lower concentration over an extended time. None of the right-sided cancers was sensitive to sodium salicylate ($P = 0.002$). *Conclusions:* Sodium salicylate had a cytostatic effect on the majority of histocultured CRC tissues. The varying chemosensitivity of the cancers possibly reflected underlying differences, for example in relation to cancer site, thus emphasizing further the usefulness of this clinically relevant system in tailoring chemotherapy to the individual patient.

Y. Liu · K.W. Eu · F. Seow-Choen · P.Y. Cheah (✉)
Department of Colorectal Surgery,
Singapore General Hospital,
Singapore 169608, Republic of Singapore
E-mail: gcscpy@sgh.com.sg
Tel.: +65-63213636
Fax: +65-62262009

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Introduction

Despite recent advances in surgical treatment strategies, the survival of colorectal cancer (CRC) patients with advanced or metastatic disease remains poor. Epidemiological and animal studies have shown that nonsteroidal antiinflammatory drugs (NSAIDs) such as aspirin can reduce the incidence of death from CRC by 50% [1, 2, 3, 4]. The antineoplastic effects of aspirin and its metabolites have also been indicated in various cancer cell lines [5, 6, 7, 8, 9]. However, different individuals often have different sensitivity or resistance to anticancer drugs. Even tumors that have been classified identically using standard histopathological criteria have been shown to respond differently to chemotherapy [10]. It is therefore necessary to develop an individualized system to determine the best drug for pre- and postoperative therapy.

We have established a relatively rapid, individualized three-dimensional histoculture system based on an adaptation of the method pioneered in other cancers by Hoffman and coworkers [11, 12] for chemosensitivity studies in primary CRC. Despite the inherent difficulty of culturing colonic tissues owing to their high bacterial content, we achieved 84% viable cultures and a contamination rate of only 10%. Histoculture systems show high preservation of in vivo tissue architecture maintaining cell-cell and cell-stroma interactions in the tumor tissues, and hence have a high predictive value for in vivo drug responses [10, 13, 14].

In this study, we used the histoculture system to explore the chemotherapeutic possibilities of sodium salicylate, an aspirin metabolite, by evaluating its cytostatic effect (i.e. inhibition of DNA synthesis) using the bromodeoxyuridine (BrdU) immunohistochemical labeling assay.

Materials and methods

Cancer specimens

The specimens used were obtained from archival tissues routinely collected from the resected colon or rectum of patients who had undergone surgery in the Singapore General Hospital. Cancer specimens were placed in DMEM/MEM within 30 min after surgery and maintained at 4°C. None of the cancers had been previously treated with chemotherapy or radiotherapy.

Histoculture

Histoculture of the cancer tissues was performed as described by Gan et al. [15]. Briefly, cancer specimens were dissected into approximately 2 mm^3 blocks or fragments, placed on a 1 cm^3 presoaked collagen gel and cultured in a humidified atmosphere of 95% air and 5% carbon dioxide at 37°C. Four to five of these fragments could be cultured on one gel in a 12-well plate for up to 1 month. The culture medium consisted of DMEM/MEM (1:1) supplemented with 9% heat-inactivated fetal bovine serum, 0.1 m*M* nonessential amino acids, 100 μg/ml gentamicin and 95 μg/ml cefotaxime. The pH of the medium was maintained at 7.4. After 4 days, the cultures were ready for experimentation.

Drug treatment

The aspirin metabolite, sodium salicylate, was prepared as a 50 m*M* stock solution in water with the pH adjusted to 7.2. Inhibition of DNA synthesis in the treated histocultures was measured in terms of the inhibition of BrdU incorporation. Cancer tissue histocultures were exposed to various concentrations of sodium salicylate ranging from 1 to 10 m*M* for 24 or 48 h. At lower concentrations, there was no apparent inhibition of the labeling index (LI). At higher concentrations (20–40 m*M*), the three-dimensional architecture of the histocultured cancer tissues was no longer maintained and there were many necrotic cells possibly due to toxicity. After washing off the drug, the histocultured fragments were incubated with 40 μ*M* BrdU for 48 h, harvested and fixed in 4% paraformaldehyde overnight, then embedded in paraffin [15].

Proliferation assay

The embedded fragments were sectioned into a consecutive series of sections of thickness 5 μm. The sections were deparaffinized and analyzed for BrdU labeling by standard immunohistochemistry methods [16]. For each treatment, at least 12 sections from different depths of each series of eight to ten fragments from duplicate experiments were scanned to identify the three fragments with the highest labeling. For each fragment, the field (as defined by a 10×10 grid) containing the highest number of labeled cells was identified and counted using ×200 magnification. These steps ensured a conservative estimate of drug response in the heterogeneous cancer [17]. The control cultures were evaluated in the same way. The validity of this approach has been verified in a recent study [18]. Typically, the total number of cells counted was in the range 200–700. The number of BrdU-labeled cells (E) in sections from treated histocultures was expressed as a percentage in relation to the number in sections from the control cultures.

Data analysis

The concentration-effect relationship was analyzed by curve fitting the experimental data to the modified Emax model as described below [19]:

$$E = E_0 [1 - C^n/(K^n + C^n)] \quad (1)$$

where E is the LI expressed as a percentage of the control value, C is the drug concentration, E_0 is the baseline effect in the absence of drug, K is the drug concentration at one-half E_0 and n is the curve shape parameter. K is equivalent to IC_{50}, the concentration needed to produce 50% inhibition. The effective plasma concentration range of salicylate for an antiinflammatory effect is 1–3 m*M* [8, 20, 21]. Since the histoculture system is an in vitro system, and the endpoint is antiproliferative rather than antiinflammatory, we arbitrarily fixed an IC_{50} of 5 m*M* (which is approximately 2.5 times the mean effective plasma concentration of salicylate for an antiinflammatory effect) as the upper limit to reflect chemosensitivity.

All statistical analyses were performed using nonparametric tests from the SPSS package. A P value of less than 0.05 was considered significant. Nonlinear regression (R^2) was carried out to check the goodness of fit for the correlation between the experimental data and the predicted value for the Emax model.

Results

Patient and cancer characteristics

A total of 20 cancer specimens from 8 male and 12 female patients were successfully cultured. The patient and cancer characteristics are summarized in Table 1. The majority of the patients (70%) were over 60 years old, which is representative of the age distribution of the colorectal cancer patients in Singapore. Cancer staging was according to Dukes' classification [22]. There were ten Dukes' A/B and ten Dukes' C/D specimens. The specimens were further classified into right or left cancers as demarcated by the splenic flexure.

Histocultures of colorectal cancers

The mean control LI of the 20 histocultures in the present study was 41 ± 14%. Table 1 lists the control LI for each of the 20 specimens. There was no significant

Table 1. Patient and cancer characteristics

Patient	Age (years)	Gender	Cancer stage	Cancer site	Control LI (%)
1	52	M	D	Left	33
2	75	F	B	Left	36
3	73	M	B	Left	31
4	69	F	B	Left	49
5	69	M	B	Left	30
6	66	F	A	Left	30
7	41	F	C	Left	29
8	83	F	D	Right	40
9	66	F	B	Left	57
10	74	M	D	Left	50
11	52	F	C	Left	20
12	69	M	D	Left	47
13	68	M	A	Left	60
14	54	F	D	Left	51
15	69	F	B	Right	40
16	57	F	C	Right	63
17	75	F	B	Right	26
18	63	M	D	Left	65
19	77	M	B	Right	20
20	42	F	C	Left	42

correlation between control LI and cancer stage, site, age or gender.

The histology of the viable regions of the histocultures (Fig. 1) was similar to that of the fresh cancer, with no observable change in tissue architecture, cell type or differentiation.

Pharmacodynamic evaluation

The relationship between sodium salicylate concentration with a 24-h exposure and inhibition of labeling was examined using histocultured cancers from 20 patients. Figure 1 shows a representative histocultured cancer with labeling decreasing as the concentration of sodium salicylate increased from 1 to 10 m*M*.

The experimental data were computer fitted to Eq. 1 according to the Emax model and values for IC_{50} and n determined for each patient. The concentration-effect curves for individual specimens revealed that the responses could be categorized into four groups. Group A was the most sensitive, groups B and C showed a sigmoidal response as predicted by the model, and group D appeared to be most insensitive to sodium salicylate (Fig. 2). The R^2 values for groups A, B and C were 0.92, 0.81 and 0.69, respectively, while that for group D was not calculated as it was obvious that there was no concentration-response relationship. Specimens from groups A and B had R^2 values more than 0.8, which suggests a good fit of the experimental values to the model-predicted values.

In total, 16 of the 20 specimens (80%) from groups A to C showed a concentration-response to sodium salicylate, suggesting that the drug has an antiproliferative effect on CRC (Fig. 2). The IC_{50} ($\pm$SE) of specimens in groups A and B were 1.2 ± 0.4 m*M* and 3.8 ± 0.5 m*M*, respectively, values within the clinically relevant concentration range [8, 19, 20]. However, specimens in group C had an IC_{50} value (35.6 ± 28 m*M*) that far exceeded 5 m*M* and therefore could not be considered sensitive to sodium salicylate for clinical purposes. Thus, 12 of the 20 specimens (60%) showed chemosensitivity to sodium salicylate within the clinically relevant concentration range.

To investigate the effect of exposure time on the concentration-response relationship of sodium salicylate, we treated six specimens (which were large enough to provide fragments for dual-time exposures) with the drug for 24 and 48 h. In specimens 9, 14, 16 and 18 (Table 2), the K or IC_{50} values decreased from 20% to 80% when the exposure time doubled. This suggests the possibility of achieving similar inhibition of proliferation with a smaller concentration of sodium salicylate and a longer exposure time. In specimen 20, interestingly, the IC_{50} value increased by 50% when the exposure time was extended, although it remained in the most sensitive group (i.e. group A). Specimen 17, as a result of a dramatic decrease in IC_{50}, was the only specimen that switched from the most insensitive group (D) to the most sensitive group (A) when the exposure time doubled. This atypical response merits further investigation.

Correlation of chemosensitivity with clinicopathological parameters

The 20 specimens were further classified into chemosensitive (groups A and B) and non-sensitive (groups C

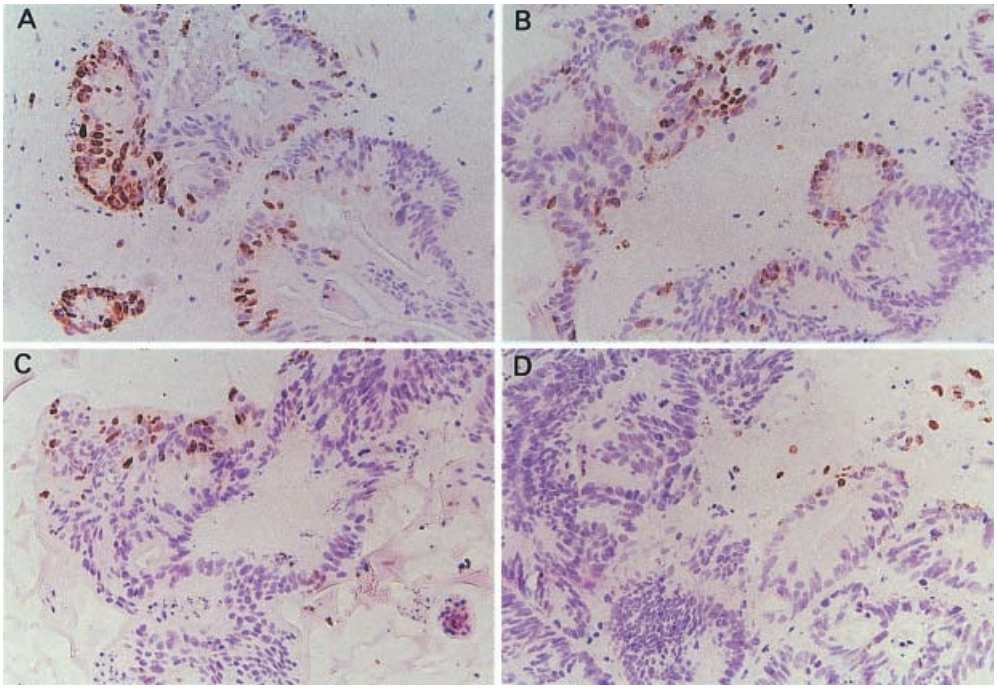

Fig. 1. BrdU immunostaining of a representative histocultured cancer tissue treated with 1 m*M* (**A**), 2 m*M* (**B**), 4 m*M* (**C**) and 10 m*M* (**D**) sodium salicylate. The mean control LI for this specimen was 42%. The mean LIs for the various treatments were 19% (**A**), 19% (**B**), 13% (**C**) and 8% (**D**). The *brown nuclei* are BrdU labeled; all nuclei were counterstained with hematoxylin (×200)

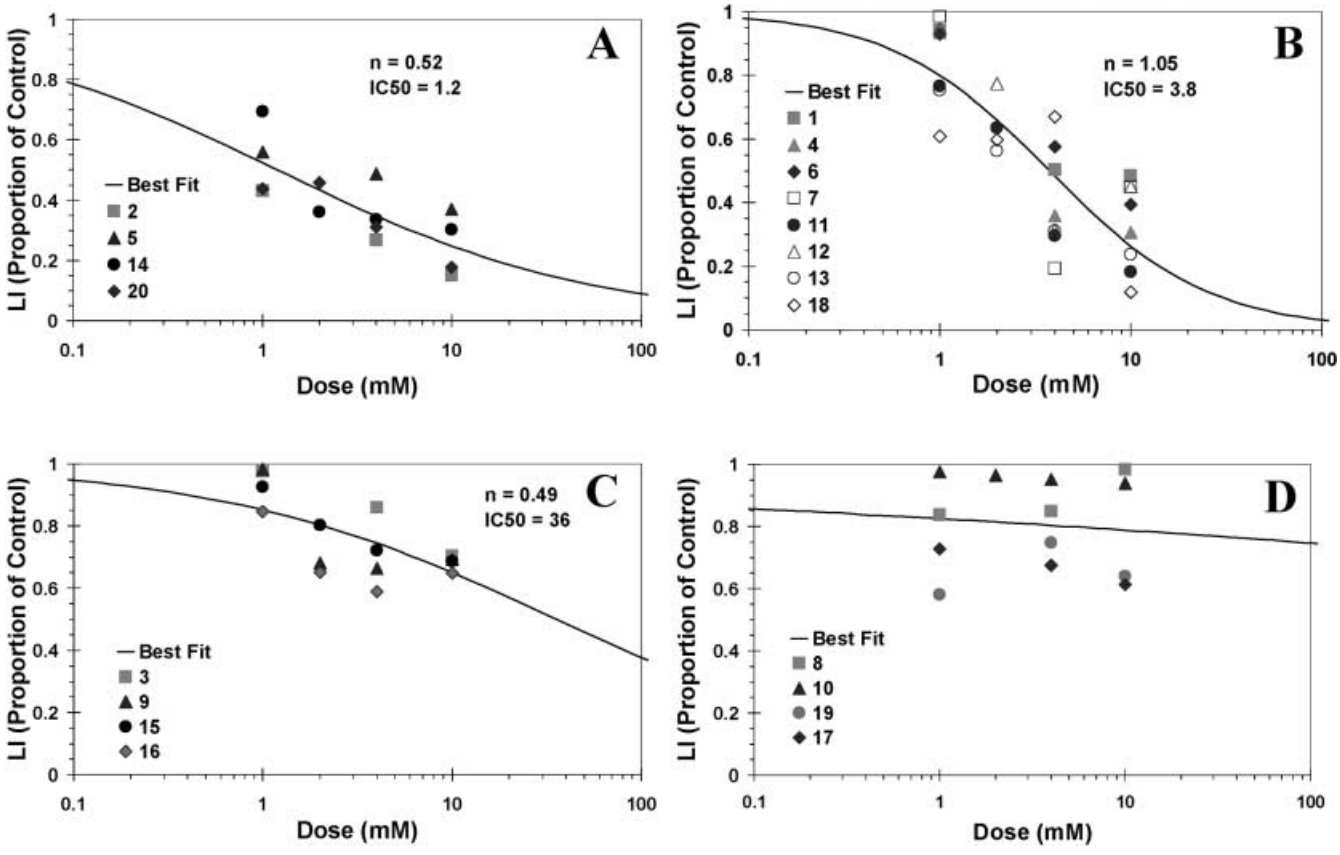

Fig. 2. Concentration-effect curves of sodium salicylate for specimens categorized into groups A–D. Group A consisted of the most sensitive specimens (**A**); group B consisted of specimens with a typical sigmoidal response (**B**); group C consisted of specimens showing a weak response (**C**) and group D consisted of the specimens least sensitive to sodium salicylate (**D**)

Table 2. Effect of exposure time on the concentration-response relationships of sodium salicylate

	Specimen no.					
	9	14	16	17	18	20
K_1 (m*M*)[a]	29.8	1.8	29.1	144.4	3.2	0.9
K_2 (m*M*)[b]	13.5	0.7	5.5	0.5	2.7	1.3
K_2/K_1	0.5	0.4	0.2	0.004	0.8	1.4

[a]K_1 is the IC_{50} after a 24-h exposure to sodium salicylate
[b]K_2 is the IC_{50} after a 48-h exposure to sodium salicylate

and D) to sodium salicylate for correlation with the clinicopathological parameters of the patients (Table 3).

None of the right-sided cancers, irrespective of patient age and gender or stage, were sensitive to sodium salicylate within the clinically relevant concentration range (Table 3; $P = 0.002$). When restricted by age group (all right-sided cancers were from the older age group), chemosensitivity to sodium salicylate was still significantly correlated with cancer site despite the reduced sample size (Fisher's exact test; $P = 0.026$). Although the younger patients (< 56 years of age) in this study appeared to be more sensitive to sodium salicylate (Table 3; $P = 0.035$), this relationship no longer held when the cancers were stratified by site (Fisher's exact test; $P = 0.505$).

There was no significant association between chemosensitivity and gender or chemosensitivity and cancer stage (Table 3).

Discussion

We report here the cytostatic effects of sodium salicylate on CRC specimens using an individualized histoculture system. The in vitro histoculture system was able to mimic the in vivo situation since the histocultured cancer tissues maintained the tissue architecture of the cancers before culture (Fig. 1) and the tissue antigenicity such as expression of the CEA antigen (data not shown). Furthermore, the LI for the control cultures as measured by BrdU incorporation in this study ($41 \pm 14\%$) was comparable to that found in an earlier study using the ^{3}H-thymidine method [23], indicating that the cancer cells were actively proliferating in this native-state histoculture.

The varying chemosensitivity to sodium salicylate (Fig. 2) highlighted the need for a clinically relevant culture system to tailor chemotherapy to the individual patient. For example, both specimens 10 and 12 were from moderately differentiated, Dukes' D sigmoid cancers in male patients with comparable ages (Table 1). However, the former was least sensitive (group D) while the latter showed a typical sigmoidal response to sodium salicylate

Table 3. Correlation between sensitivity to sodium salicylate and clinicopathological parameters

Parameter	No. of patients (%)	Sensitivity		*P* value
		Yes (%)	No (%)	
Gender				
Male	8 (40)	5 (63)	3 (37)	0.852
Female	12 (60)	7 (58)	5 (42)	
Age group (years)				
< 56	5 (25)	5 (100)	0 (0)	0.035
≥56	15 (75)	7 (47)	8 (53)	
Cancer stage (Dukes')				
A/B	10 (50)	5 (50)	5 (50)	0.833
C/D	10 (50)	7 (70)	3 (30)	
Cancer site				
Left	15 (75)	12 (80)	3 (20)	0.002
Right	5 (25)	0 (0)	5 (100)	

(group B; Fig. 2). The differences in sensitivity could possibly have been due to genetic differences which could be further investigated using the individualized histoculture system. Such differences cannot be easily studied in cell lines since these may lose or acquire new lesions compared to the original cancers. Furthermore, the responses of xenografts are not always representative of the response in humans. In some instances, xenograft tumors behave more like mouse tumors than human tumors [24]. Therefore, effective human drugs may be missed if a xenograft model is used.

Initial analysis with several specimens showed the possibility of achieving a similar inhibitory effect with a lower dose of sodium salicylate by increasing the exposure time (Table 2). Nevertheless, for all but one specimen (specimen 17), doubling the exposure time did not appear to alter the sensitivity to sodium salicylate, i.e. if the response of a particular tissue specimen belonged to group C (non-sensitive), it remained in group C.

In addition, the chemosensitivity to sodium salicylate could be correlated with the clinicopathological parameters of the patients (Table 3). It is as yet unclear why all the right-sided cancers in this study were insensitive to sodium salicylate. Additional study with a larger series is required to confirm this observation, although it could possibly reflect underlying genetic differences in the pathogenesis of right- and left-sided cancers [25, 26, 27]. When stratified by site, chemosensitivity was no longer significantly correlated with patient age, suggesting that the significant relationship probably merely reflected the fact that there are more right-sided cancers among older patients [27]. No significant association between chemosensitivity and gender was found, indicating that no sex-associated genes contribute to chemosensitivity to sodium salicylate. A prospective study has also shown that the decreased risk of digestive tract cancers is similar in men and women with frequent and prolonged use of aspirin [28].

Another mechanism of action of aspirin and its metabolites could be the induction of cell death. To assess whether sodium salicylate induced apoptosis in our system, we stained the slides with a new anti-Poly(ADP-ribose) polymerase (PARP) p85 fragment antibody (Promega, Madison, Wis.). Anti-PARP p85 antibody detects a caspase-3-cleaved 85-kDa fragment of PARP, a hallmark of apoptosis [29]. Although apoptosis was clearly detectable in staurosporine-treated (positive control) sections, our preliminary data did not reveal a concentration-dependent increase in apoptotic index in sodium salicylate-treated sections compared to control sections, leading us to conclude that sodium salicylate probably did not induce caspase-activated apoptosis in the histoculture system (data not shown). The role of apoptosis in aspirin-induced cell death remains controversial. Although some studies [6, 30] have shown that aspirin induces apoptosis in some colon cancer cell lines, others have shown that aspirin does not induce apoptosis [5, 31] or induces cell death via necrosis rather than apoptosis [7].

The predictive value of an in vitro chemosensitivity assay is validated only by prospective clinical trials. In a correlative clinical trial involving 16 colorectal cancer patients [32], a 100% correlation was found between drug response in the histoculture assay and clinical outcome. That is, patients whose tumors were evaluated as sensitive in the in vitro assay responded to the drug and vice versa. There was no false-positive or false-negative case in the series. Although the endpoint and drug evaluated in the study were different from those of the present study, the in vitro assay systems are essentially similar, lending unequivocal support to the use of the histoculture system for predicting chemosensitivity of an individual patient.

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