in vivo *19*: 31-36 (2005)

Phenolic Compounds, Sodium Salicylate and Related Compounds, as Inhibitors of Tumor Cell Growth and Inducers of Apoptosis in Mouse Leukemia L1210 Cells

ANN H. CORY and JOSEPH G. CORY

Department of Biochemistry and Molecular Biology, Brody School of Medicine, East Carolina University, Greenville, NC 27834, U.S.A.

Abstract. *The effects of a series of phenolic compounds were compared to the effects of sodium salicylate (2-hydroxybenzoate) on the growth, cell cycle and apoptotic effects in wild-type (WT) and deoxyadenosine-resistant (Y8) L1210 leukemia cells. These compounds included: salicylaldehyde, salicylaldoxime, salicylhydroxamic acid, salicylamide, 5-aminosalicylate and 5-sulfosalicylate. The IC_{50} values for inhibition of tumor cell growth ranged from 40 μM for salicylaldhyde to greater than 4 mM for 5-sulfosalicylate. There appeared to be an excellent correlation between the IC_{50} value for a compound and the ratio of octanol/aqueous distribution. Salicylamide caused a G2/M block in both the WT and Y8 L1210 cells, while salicylalehyde caused a G0/G1 block in both the WT and Y8 cells. Salicylamide and salicylaldoxime caused a much greater apoptotic effect in the Y8 cells than in the parental WT L210 cells. These data suggest that salicylaldehyde and salicylaldoxime, the most active compounds in this series, may provide the lead chemicals from which other more active drugs can be synthesized.*

Previous studies have shown that an L1210 cell line (Y8) selected for resistance to deoxyadenosine targeted at the ribonucleotide reductase (RR) site had alteration(s) that led the RR in these cells to be insensitive to dATP as a potent inhibitor of ribonucleotide reductase activity. However, the Y8 cells also had other phenotypic alterations that could not be overtly attributed to RR (1-3). One of the major changes seen in the Y8 cells in response to a variety of agents was that the Y8 cells became apoptotic under conditions in which the parental wild-type (WT) cells did not apoptose. This included agents such as radiation (4), doxorubicin (5), and roscovitine (6), which are summarized in a review (3). In each case of drug-induced apoptosis, with the exception of Gemcitabine-induced apoptosis, it was found that caspase-3 activation was part of the apoptotic response.

The previous studies also included determination of the effects of sodium salicylate on the WT and Y8 L1210 cells (7). It was found that sodium salicylate caused a marked increase in the fraction of apoptotic cells in the Y8 cell population that was not seen in the parental WT L1210 cells. In the current report, we present data that compare the effects of other 2-hydroxyphenolic compounds on the WT and Y8 L1210 cells with respect to cell growth, cell cycle and apoptosis. These studies show that there are differences not only in the IC_{50} values, cell cycle and apoptotic effects among the compounds, but also between the WT and Y8 L1210 cell lines. Salicylaldehyde and salicylaldoxime may offer new lead compounds that can be further developed as potentially more active phenolic compounds than sodium salicylate.

Correspondence to: Joseph G. Gory, Department of Biochemistry and Molecular Biology, Brody School of Medicine, East Carolina University, Greenville, NC 27834, U.S.A.

Key Words: Phenolic compounds, sodium salicylate, apoptosis.

Materials and Methods

Growth of mouse leukemia L1210 cells. The parental wild-type L1210 cells were originally purchased from the American Type Tissue Collection, Rockville, MD, USA. The deoxyadenosine-resistant (Y8) L1210 cells were selected for resistance to deoxyadenosine as previously described (8). The cells were grown in RPMI 1640 culture medium supplemented with 10% horse serum, $NaHCO_3$ (2g/l), and gentamycin (50mg/l) and maintained at 37° C in a humidified atmosphere (95% air/5% CO_2). The cells were subcultured every two-to-three days to maintain the cells in log-phase.

Inhibition of cell growth. To WT and Y8 cells in log-phase, various concentrations of the 2-hydroxyphenolic acid analogs were added. The pH of each drug solution was adjusted to pH 7 before addition to the cell cultures. In each experiment, five different concentrations of drug in triplicate were set up for growth inhibition studies. Each experiment was repeated at least twice with different batches of WT and Y8 L1210 cells. After 72 hours of incubation in the presence of drug, aliquots of the cells were taken for cell counts using a Coulter Counter, model ZI.

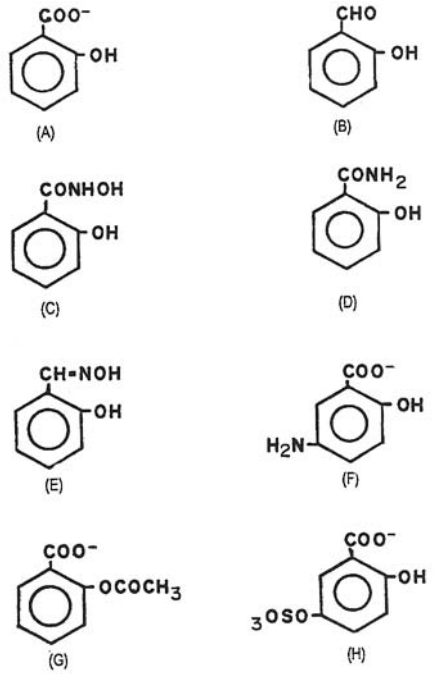

Figure 1. *Structures of 2-hydroxybenzoate compounds. A. sodium salicylate (2-hydroxybenzoate); B. salicylaldehyde (2-hydroxybenzaldehyde); C. salicylhydroxamic acid (2-hydroxyhydroxamic acid); D. salicylamide (2-hydroxybenzamide); E. salicylaldoxime (2-hydroxybenzaldehyde oxime); F. 5-aminosalicylate (2-hydroxy-5-aminobenzoate); G. acetylsalicylate (aspirin) and H. 5-sulfosalicylate (2-hydroxy-5-sulfobenzoate).*

Cell cycle analysis by flow cytometry. The WT and Y8 cells were treated with the 2-hydroxyphenolic compounds for 24 hours. Using the method of Krishan (9), the cells (aliquots of 1.0 x 10^6 cells in duplicate) were stained with propiduim iodide and kept overnight at 4˚C. Prior to running the samples on a Becton-Dickinson FACSCAN (San Jose, CA, USA), the cells were passed through a 25-gauge needle. Ten thousand events were recorded for each sample and the data analyzed using BD Modfit software. All experiments were carried out at least twice.

Measurement of apoptosis by flow cytometry. WT and Y8 cells, in log-phase, were treated with the drugs for 24 hours. Duplicate aliquots of cells (1.0 x 10^6 cells) were collected by centrifugation and washed with cold phosphate-buffered saline. Apoptosis was determined using the Annexin-V-FLUOS kit (Boehringer-Mannheim, Indianapolis, IN, USA). The necrotic fraction was determined simultaneously by the uptake of propidium iodide. The cells were analyzed on a BD FACSCAN, using 488 nm excitation, a 515 nm bandpass filter for fluorescein detection and a 560 nm filter for propidium iodide detection. Ten thousand events were collected for each sample. BD Lysis II software was used to analyze the data. Each experiment was carried out at least twice with different batches of cells.

Distribution of 2-hydroxybenzoate compounds between the n-octanol and aqueous layer. Aliquots of solutions of the compounds were added to a mixture of equal volumes of n-octanol and phosphate-buffered saline. The samples were mixed vigorously on a vortex mixer and centrifuged. Aliquots of the n-octanol phase and the aqueous phase were taken for measurement of absorbance at 325 nm.

Table I. *Effect of phenolic compounds on the growth of wild-type and deoxyadenosine-resistant L1210 cells.*

	IC_{50}, μM [b]	
Compound [a]	WT	Y8
salicylaldehyde	40	46
salicylaldoxime	85	101
5-aminosalicylate	980	1,000
salicylamide	1,130	1,380
acetylsalicylate	1,580	1,850
salicylate	1,110	2,240
5-sulfosalicylate	>4,700	>4,700

[a] the pH of the solutions containing these compounds was adjusted to pH7 before adding to the culture medium
[b] average of two separate determinations

Results

Effect of 2-hydroxybenzoate compounds on WT and Y8 L1210 cell growth in culture. As seen in Figure 1, a series of compounds having the general structure related to 2-hydroxybenzoic acid (with the exception of aspirin, 2-acetylbenzoic acid) were studied for their effects on the growth of the WT and Y8 L1210 cells in culture. As seen in Table I, salicylaldhyde was the most active compound in terms of inhibiting L1210 cell growth in culture and there was essentially no difference in the IC_{50} values between the WT and Y8 cells. The IC_{50} values ranged from 40 μM (salicylaldehyde) to greater than 4,700 μM for 5-sulfosalicylate. The IC_{50} values determined for these compounds correlated extremely well with the apparent ability of the drugs to enter the cell as estimated by the ratio of drug in the n-octanol phase to the aqueous phase (Table II).

Effect of 2-hydroxybenzoate compounds on the cell cycle of WT and Y8 L1210 cells. The 2-hydroxybenzoate compounds were added to the WT and Y8 cells in log-phase. After 24 hours, the control and drug-treated cells were collected and analyzed by flow cytometry to determine the distribution of the cells in the phases of the cell cycle. As seen in Figure 2 and quantitated in Table III, salicylamide induced a strong

Table II. *Distribution of the phenolic compounds between n-octanol and aqueous phases.*

Compound	Octanol phase/aqueous phase [a]
salicylaldehyde	36
salicylaldoxime	64
5-aminosalicylate	0.004
salicylamide	4
salicylate	0.11
sulfosalicylate	0.002

[a] a standard solution of the phenolic compound was added to equal volumes of phosphate-buffered saline and n-octanol; the samples were vigorously mixed on a vortex mixer and centrifuged. The absorbance of the n-octanol and aqueous phases were determined.

Table III. *Effect of 2-hydroxyphenolic compounds on the cell cycle distribution of wild-type and deoxyadenosine-resistant L1210 cells.*

		% distribution [b]		
Cell type	Compound [a]	G0/G1	S	G2/M
WT	control	40	47	13
	salicylaldhyde	53	39	8
	salicylaldoxime	33	59	7
	salicylamide	8	61	31
Y8	control	46	45	9
	salicylaldehyde	62	27	10
	salicylaldoxime	53	33	13
	salicylamide	40	21	38

[a] WT and Y8 cells in log-phase were incubated with drug for 24 hours.
[b] Cell cycle analyses were run as described in the "Methods" section.

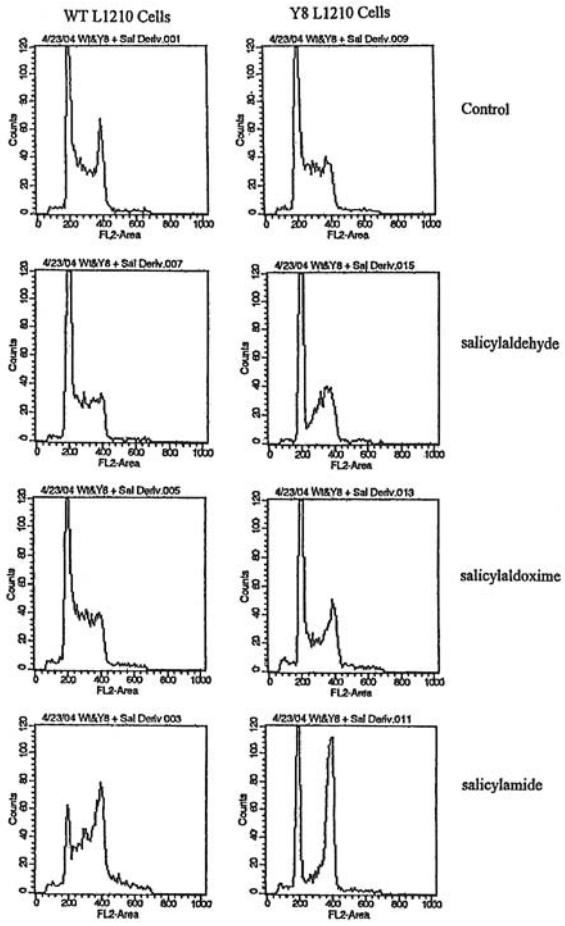

Figure 2. *Effects of 2-hydroxyphenolic compounds on the cell cycle of wild-type and deoxyadenosine-resistant L1210 cells. WT and Y8 L1210 cells were incubated in the presence of the 2-hydroxybenzoate compound for 24 hours. Aliquots of cells were taken in duplicate for cell cycle analysis by flow cytometry using propidium iodide staining. The data are the average of two separate experiments using different batches of cells.*

G2/M cell cycle block in both the WT and Y8 cells. However, the WT cells accumulated in the S-phase population at the expense of the G0/G1 population. This S-phase accumulation was not seen in the Y8 cells. Salicylaldoxime caused an S-phase accumulation in the WT cells, but not in the Y8 cells while salicylaldehyde caused a modest G0/G1 cell cycle block in both the WT and Y8 cells.

Effect of 2-hydroxybenzoate compounds on apoptosis induction in WT and Y8 L1210 cells. Since previous studies had shown that the WT and Y8 cells responded differently to sodium salicylate with respect to an apoptotic response, the effects of the various 2-hydroxybenzoate compounds on apoptosis induction in the WT and Y8 L1210 cells were determined. As seen in Figure 3 and quantitated in Table IV, salicylaldoxime and salicylamide induced significant apoptosis with limited necrosis in the Y8 cells, but did not induce apoptosis in the WT cells. The level of apoptosis induced by salicylaldehyde was minimal in both the WT and Y8 L1210 cell lines.

Discussion

In a series of reports, it has been shown that sodium salicylate induces apoptosis in a variety of cell types (10-12). In our studies, we had shown that sodium salicylate induced apoptosis in deoxyadenosine-resistant (Y8) mouse leukemia L1210 cells, but did not cause apoptosis in the parental wild-

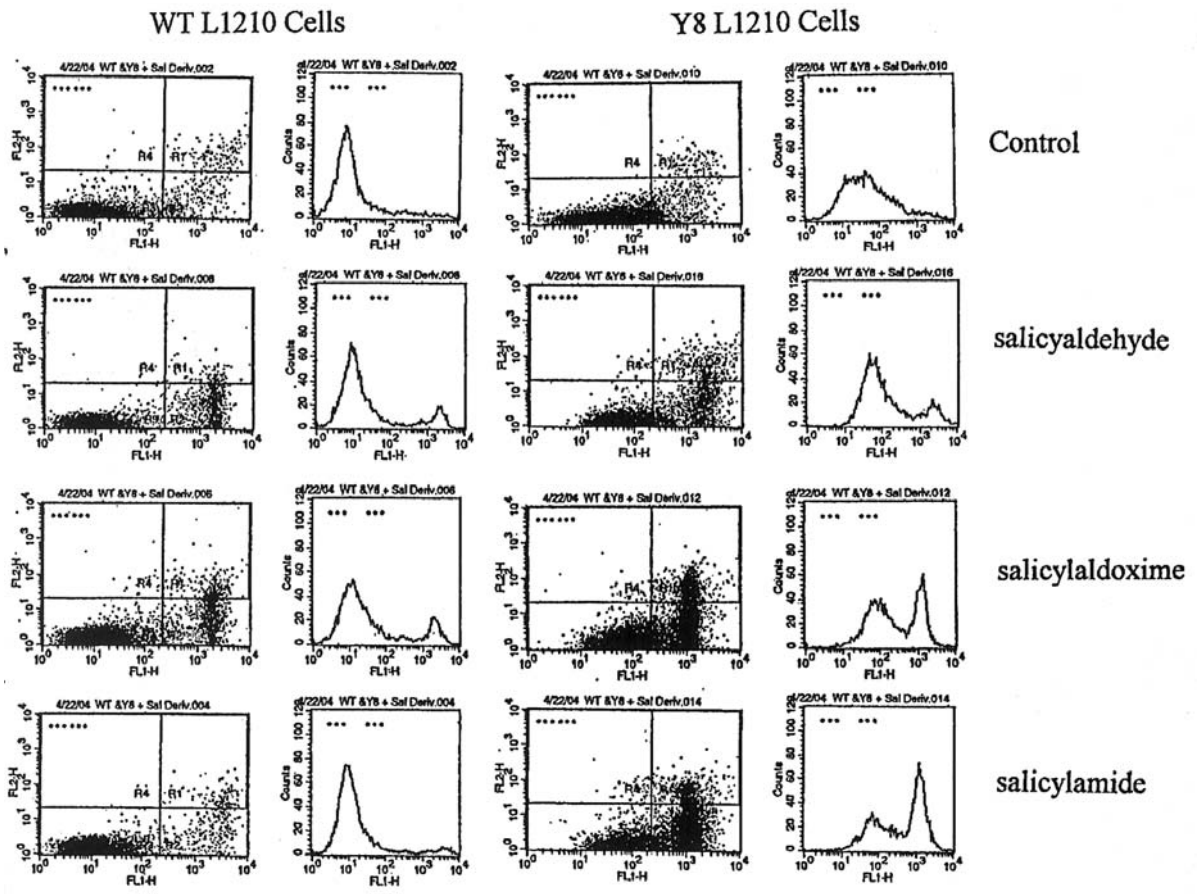

Figure 3. *Effects of 2-hydroxyphenolic compounds on apoptosis in wild-type and deoxyadenosine-resistant L1210 cells. WT and Y8 cells were incubated in the presence of the 2-hydroxyphenolic compound for 24 hours. Aliquots of the cells were taken in duplicate for determination of the apoptotic and necrotic cells from the average of two separate experiments using different batches of cells.*

Table IV. *Effect of 2-hydroxyphenolic compounds on apoptosis induction in wild-type and deoxyadenosine-resistant L1210 cells.*

		%fraction	
Cell type	Compound	apoptotic	necrotic
WT	control	4.2	1
	salicylaldehyde	10.0	1.0
	salicylaldoxime	17.0	3.9
	salicylamide	3.8	1.5
Y8	control	9.4	1.9
	salicylaldehyde	18.4	3.4
	salicylaldoxime	46.8	5.0
	salicylamide	41.6	9.1

type (WT) L1210 cells. The WT L1210 cells express mutant p53 while the Y8 cells do not express p53 (1, 2). It was shown that sodium salicylate-induced apoptosis in the Y8 cells proceeded *via* caspase-3 activation (7). Other studies have shown that treatment of cells with sodium salicylate leads to multiple effects, that may or may not be related, that result in apoptosis. These include effects on the MAPK pathway (13, 14), ROS activation (15), induction of hsp genes (16) and suppression of COX-2 levels (17).

In the studies reported here, the effects of a series of 2-hydroxybenzoate compounds were studied for their effects on tumor cell growth, cell cycle effects and induction of apoptosis in the wild-type and deoxyadenosine-resistant L1210 cells. It was found that salicylaldehyde was most active at inhibiting tumor cell growth in culture (Table I). Salicylaldehyde was approximately 40-times more active than sodium benzoate in terms of inhibiting the growth of both the WT and Y8 cells (IC_{50} approx.40 μM). The other compounds in the series had IC_{50} values, of approximately

1000-2000 μM, with the exception of 5-sulfosalicylate which had an IC_{50} value in excess of 4,700 μM. As shown in Table II, there was an excellent correlation between the IC_{50} value for a compound and the ratio of distribution between the n-octanol and aqueous phases suggesting that the ability of the drug to enter the cell greatly enhanced its ability to inhibit tumor cell growth.

The data showed that there were significant differences not only between the compounds, but also between the WT and Y8 cell lines. Salicylamide caused a strong G2/M cell cycle block in both the WT and Y8 cells with an accumulation of S-phase cells in the WT cells, while salicylaldehyde caused a G0/G1 block in both cell lines. Salicylaldoxime caused an S-phase block in the WT cells, but there was not a corresponding S-phase block in theY8 cells.

When these same compounds were studied for their effects on induction of apoptosis in the WT and Y8 cells, it was found that there was a marked difference in the apoptotic responses. Salicylaldoxime and salicylamide caused a large fraction (50%) of the Y8 cells to become apoptotic and necrotic under conditions in which less than 20% of the WT cells became apoptotic.

These studies show that 2-hydroxybenzoate compounds having some structural relationship to sodium salicylate may provide insight into lead compounds that could be further developed for cancer chemotherapy. At this time, it is not known if these compounds act through the same mechanism(s) that have been shown for sodium salicylate. The difference in the cell cycle and apoptotic responses suggest that different pathways may be altered by these drugs. Future studies will be carried out in this direction.

References

1 Cory JG, Cory AH, Long SD, Carter GL and Johnson CE: Altered steady-state levels of the messenger RNAs for c-myc and p53 in L1210 cell lines selected for resistance to deoxyadenosine. Cancer Res *52*: 2000-2003, 1992.

2 Cory JG, He A-W and Cory AH: Deoxyadenosine-resistant mouse leukemia L1210 cell lines with alterations in early response genes and p53. Anticancer Res *16*: 3483-3490, 1996.

3 Cory AH, Edwards CC, Hall JG and Cory JG: Inhibitors of diverse metabolic steps cause increased apoptosis in deoxyadenosine-resistant mouse leukemia L1210 cells that lack p53 expression: convergence at caspase-3 activation. Advan Enzyme Regul *43*: 29-45, 2003.

4 He A-WR and Cory JG: Effect of ionizing radiation on wild-type and mutant mouse leukemia L1210 cells. Oncology Res *10*: 455-464, 1998.

5 He A-WR and Cory JG: Effect of doxorubicin on wild-type and deoxyadenosine-resistant mouse leukemia L1210 cells. Int J Oncology *14*: 891-895, 1999.

6 Somerville L and Cory JG: Enhanced roscovitine-induced apoptosis is mediated by a caspase-3-like activity in deoxyadenosine-resistant mouse leukemia L1210 cells. Anticancer Res *20*: 3347-3356, 2000.

7 Hall JG, Cory AH, Hickerson DHM and Cory JG: Increased sensitivity to sodium salicylate-induced apoptosis in drug-resistant leukemia L1210 cells. Anticancer Res *21*: 173-180, 2001.

8 Carter GL and Cory JG: Selective resistance of L1210 cell lines to inhibitors directed at the subunits of ribonucleotide reductase. Advan Enzyme Regul *29*: 123-129, 1989.

9 Krishan A: A rapid flow cytofluorometric analysis of mammalian cell cycle by propidium iodide staining. J Cell Biol *66*: 188-193, 1975.

10 Schwenger P, Bellosta P, Vietor I, Basilico C, Skolnik EY and Vilcek J: Sodium salicylate induces apoptosis *via* p38 mitogen-activated protein kinase but inhibits tumor necrosis factor-induced c-jun N-terminal kinase/stress-activated protein kinase activation. Proc Natl Acad USA *94*: 2869-2873, 1997.

11 Bellosillo B, Pegrie M, Barrragan M, Castano E, Villamor N, Colomer D, Montserrat E, Pons G and Gil J: Aspirin and salicylate induce apoptosis and activation of caspase in B-cell chronic lymphocyte leukemia cells. Blood *92*: 1406-1414, 1998.

12 Klampfer L, Cammenga J, Wisniewski H-G and Nimer SD: Sodium salicylate activates caspases and induces apoptosis of myeloid leukemia cell lines. Blood *93*: 2386-2394, 1999.

13 Vittimberga FJ, Jr, McDade TP, Perugini RA and Callery MP: Sodium salicylate inhibits macrophage TNF-α production and alters MAPK activation. J Surgical Res *84*: 143-149, 1999.

14 Lee EJ, Park HG and Kang HS: Sodium salicylate induces apoptosis in HCT 116 colorectal cancer cells through activation of p38 MAPK. Int J Oncology *23*: 503-508, 2003.

15 Chung YM, Bae YS and Lee SY: Molecular ordering of ROS production, mitochondrial changes, and caspase actvation during sodium salicylate-induced apoptosis. Free Radical Biol Med *34*: 434-442, 2003.

16 Ishihara K, Horiguchi K, Yamagishi N and Hatayama T: Identification of sodium salicylate as an hsp inducer using a simple screening system for stress response modulators in mammalian cells. Env J Biochem *270*: 3461-3468, 200.

17 Kavon KS and Chae HJ: Sodium salicylate inhibits expression of COX-2 through suppression of ERK and subsequent NFkappa B activation in rat ventricular cardiomyocytes. Arch Pharm Res *26*: 545-553, 2003.

Received November 1, 2004
Accepted November 19, 2004