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Novel Resveratrol-based Aspirin Prodrugs: Synthesis, Metabolism, and Anticancer Activity

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Running Title: Novel Resveratrol-based Aspirin Prodrugs

ABSTRACT

Regular aspirin use has been convincingly shown to reduce the risk of colorectal cancer. However, long-term use of aspirin leads to gastrotoxicity. Herein, we designed and synthesized a novel class of resveratrol-based aspirin prodrugs to simultaneously release aspirin and resveratrol to attenuate the side effects caused by aspirin. Prodrug RAH exerted enhanced anticancer activities which are better than a physical mixture of aspirin and resveratrol as well as each individual. Metabolism of RAH in mice showed that the majority of RAH is decomposed to release resveratrol and aspirin or salicylic acid either in the intestine or after absorption. Mechanistic studies demonstrate RAH inhibits cell cycle arrest through downregulation of cyclins, and induces apoptosis by activation of caspase-3 in cancer cells. These findings highlighted the improved anticancer properties of resveratrol-based aspirin prodrugs. RAH may represent novel and safe alternatives of aspirin for the purpose of daily use in the future.

INTRODUCTION

Colorectal cancer (CRC) is the third most common form of cancer and the fourth most frequent cause of cancer deaths worldwide.[1] Most cases of CRC are sporadic, and genetic and environmental factors play important roles. Aspirin (ASA) is a classic wonder drug and has been widely used for the treatment of inflammation, fever, and pain over a century.[2] Recently, ASA has received another boost due to its potential benefits in the prevention/treatment of cancers and cancer prevention.[3-5] The main targets of ASA are cyclooxygenase (COX) isoforms, COX-1 and COX-2, which catalyze the rate-limiting step in the conversion of arachidonic acid to prostaglandins (PGs).[6] PGs play a key role in the generation of the inflammatory response. COX-1, expressed constitutively in most tissues, is the dominant source of PGs that have a protective effect on gastric mucosa.[7] Inhibition of COX-1 impairs the synthesis of PGs, and adverse effects occur as a result of effects on the constitutively expressed COX-1.[8] COX-2, induced by inflammatory stimuli, hormones, and growth factors, is mainly responsible for inflammatory reactions and tumor developments.[9] Long-term use of ASA is limited because of its lack of COX-2 selectivity, which is generally considered as the main drawback of ASA and accounts for ASA's main side effects, such as gastric ulceration.[8] Gastrointestinal toxicity can be reduced by selective inhibitors of COX-2,[10] but chronic use leads to an increased risk of heart attack and stroke.[11] Other than COX-2 inhibitors, significant efforts have been devoted to develop nitric oxide-releasing ASA derivatives in order to better control side effects of nonsteroidal anti-inflammatory drugs (NSAIDs). Nitric oxide (NO) is an important gaseous messenger that mediates a variety of physiological actions, including gastroprotetction.[12] Release of NO in the gastrointestinal tract (GIT) would lead to a local relaxation of the blood vessel with lower adhesion of leukocytes, therefore improving protection of the gastric mucosa and reducing

haemorrhagic effects.[13] Positive *in vitro* and *in vivo* results are available,[14-16] but long-term use of organic nitrate derivatives of NO-ASA can lead to nitrate tolerance, as occurs with nitroglycerine.[17] In addition, some new-developed NO-ASA derivatives were demonstrated to release NO with expulsion of formaldehyde,[18] the latter has been identified as a human carcinogen recently.[19] Hence, the advancement of new ASA derivatives with more favorable and safer profiles would be a welcome development indeed.

Various plant polyphenols have been shown to have a therapeutic potential for the effective treatment of peptic ulcers.[20] Resveratrol (RES) is without a doubt the most famous dietary polyphenol present in peanuts, various berries, grapes and red wine, and has been reported to be capable of protecting the gastric mucosa against the NSAID-induced side effects.[21, 22] Besides its promising activities against inflammation, heart disease and aging,[23] numerous preclinical findings consider RES as an up-and-coming natural arsenal for cancer prevention and treatment.[24, 25] Clinic trials of RES in CRC patients further added RES reduces tumor cell proliferation.[26] Daily oral administration of a high dose of RES, up to 750 mg/kg, to rats for 90 days demonstrated the nontoxicity of this polyphenol.[27, 28] Moreover, recent clinical trials evidenced that no adverse events were reported to resveratrol supplementation at doses < 0.5 g/day when the duration was short.[29] Thus, RES could be an ideal additive to the clinic use of ASA to ameliorate the side effects caused by ASA.

A combination of ASA and RES can be an attractive strategy to retain anticancer and anti-inflammatory properties of ASA while reducing gastric toxicity. Major concerns in joint use include drug interactions, the definitive exposure to the desired targets, and individual pharmacokinetics and biodistribution parameters. These difficulties could be overcome by constructing a single prodrug containing these two moieties, which is able to be decomposed to

release corresponding individuals after administration. However, little is known for the formation of natural product-based ASA derivatives with gastroprotection so far. With these challenges in mind, we constructed a class of RES-based ASA derivatives (**4-10**) (Scheme 1), with the ability to release ASA and RES for their respective biological properties.

RESULTS AND DISCUSSION

Chemical Synthesis. Acylation of RES (**1**) by *O*-acetylsalicyloyl chloride in the presence of Et_3N at 0 ^oC produced a RES-ASA hybrid (RAH) **4** and its regioisomer **5** with a ratio of around 2.3:1. Likewise, coupling reaction of pterostilbene (**2**), a dimethylated RES, with *O*-acetylsalicyloyl chloride afforded a pterostilbene-ASA hybrid **6** in 96% yield. Hydrolysis of **6** by dilute HCl solution led to a deacetylated product **7**. Prudent treatment of **6** by BBr_3 in CH_2Cl_2 at -78 ^oC produced prodrug **8** in 3% yield, otherwise demethylated products **9** and **10** were harvested as major products at 0 ^oC (Scheme 1).

In order to validate the biological properties of the molecular skeleton composed of ASA and RES, two RAH-like molecules **11** and **12**, which have the molecular skeleton consisted of ASA residue and RES moiety, were synthesized (Scheme 2). RAH resemblances, **11** and **12**, are more structurally stable, insusceptible to breakage, and most likely exerting their own biological effects as whole molecules. Analogue **11** was synthesized as outlined in Scheme 2A. In brief, 4-bromobenzyl bromide was first refluxed with PPh_3, and a subsequent Wittig reaction with 3,5-dimethoxybenzaldehyde in the presence of $NaOBu^{-t}$ provided 4-bromostilbenoid **14** in 66% overall yield. Bromostilbene **14** was treated with salicylaldehyde in the presence of of n-BuLi to give additive product **15**. *Ortho*-hydroxy assisted and copper-catalyzed oxidation of compound **15** in DMF produced ketone **16**,[30] which was converted to the target compound **11** by BBr_3 in 62% yield. The synthetic route for resemblance **12** was shown in Scheme 2B. In detail, reductive

coupling of 4-bromobenzyl bromide with *O*-acetylsalicyloyl chloride in the presence of $Pd(PPh_3)_4$ and zinc produced coupling product **19**. Removal of acetyl group in **19** led to deacetylated product **20** in 96% yield.[31] A Heck reaction between **20** and **18** in the presence of $Pd(PPh_3)_4$ and Et_3N provided coupling product **21** in 14% yield.[32] Deacetylation of **19** is the key process to succeed in the preparation of intermediate **21**, otherwise acetyl group would be further involved in an intermolecular aldol reaction (data not shown). Finally, demethylation of **21** took place using BBr_3 to furnish the target compound **12**.

In Vitro Cytotoxic Activity. RES-based ASA derivatives **4-10** were evaluated for their anti-cancer activities against human colon cancer cells HCT-116 and HT-29 using MTT assay and were compared with the reference compounds RES (**1**), pterostilbene (**2**) and ASA (**3**). As shown in Figure 1, prodrugs **4-10** were more active than the individual drugs **1-3**. In particular, RAH **4** displayed the most potential in both cancer cell lines, and followed by deacetyl RAH (DA-RAH) **10**. RAH **4** was more active than dimethylates **6** and **7**, as well as mono-methylates **8** and **9**, indicating that the two free hydroxyl groups at C-3 and C-5 in the ring A of RES moiety play an important role in the observed activity. In addition, RAH **4** exhibited a better activity than its regioisomer **5**, indicating the manner in which the two moieties are linked in molecule **4** favors the anticancer activity.

As the most potent RES-ASA hybrid, RAH **4** was further compared to an equimolar mixture of ASA and RES (ASA+RES) using colony formation assay (Figure 2). Our results showed that RAH is much more active than ASA+RES in both human colon cancer cells in a dose-dependent manner (Figure 2A). These observations indicate that the molecular skeleton composed of ASA and RES possesses antiproliferative properties. In order to validate the hypothesis mentioned above, we further evaluated the anticancer properties of two RAH-like molecules **11** and **12**. Our

results showed that both RAH-like molecules **11** and **12** exert comparable anti-proliferative activities to that of RAH in both cell lines (Figures 1 and 2B). Interestingly, molecule **11** is more potent in inhibiting the formation of colonies than its resemblance **4** in both cells, while molecule **12** elicits parallel ability suppressing the growth of colonies to RAH **4** in HT-29. These findings further validated the molecular skeleton consisted of ASA and RES has anticancer properties. As a consequence, we demonstrated that 1) free hydroxyl groups in the ring A of the RAH moiety play an important role in activity; 2) RAH is more active than an equimolar mixture of ASA and RES, individual ASA, or individual RES; and 3) the molecular skeleton composed of ASA and RES has anticancer properties.

Metabolism of RAH in Cancer Cells. We have identified RAH **4** as the most promising anticancer prodrug containing a combination of ASA and RES, its ability to release RES and ASA was studied in cancer cells HCT-116 using HPLC-coulometric electrode array detector (CEAD) (Figure 3). Our results illustrated that RAH was gradually hydrolyzed into deacetyl RAH (DA-RAH) **10** in the first 2 h of incubation, and then DA-RAH was accumulated correspondingly to a maximum at around 4 h. Next, DA-RAH was gradually decomposed to release RES and salicylic acid (overlapped by background) over 24 h. This indicated that antiproliferative activity of RAH in cancer cells may reasonably be attributed to intact RAH and DA-RAH as well as individual RES and salicylic acid.

Major Metabolites and Distribution of RAH in Mice. The metabolism and biodistribution of RAH **4** in mice was further investigated using LC-ESI tandem mass spectrometry. As a result, fourteen metabolites (**1**, **10**, and **22–33**) were identified in mouse GIT contents, colon tissue, plasma, urine or feces after intragastric RAH administration (Table 1 and Figure 4). Compounds **1**, **4**, **10**, and **22–24** were fully identified by direct comparison with the available standards. The

rest of metabolites were tentatively identified according to their MS/MS spectra (Table 1). In brief, compound **25** had a molecular ion at *m/z* 307 $[M - H]^-$ (227 + 80), indicating **25** is the sulfate metabolite of RES. This was further confirmed by observing *m/z* 227 $[M - 80 - H]^-$ (loss of one sulfate moiety from *m/z* 307) as the major product ion in its MS/MS spectrum (Table 1). This was also supported by non-conjugated RES at *m/z* 227 $[M - H]^-$ found in mouse urine after enzymatic hydrolysis (Figure 4). Peak **26**, with a molecular ion at *m/z* 579 $[M - H]^-$ (227 + 176 × 2), had a major MS/MS fragment at *m/z* 403 $[M - 176 - H]^-$ (loss of one glucuronide moiety from *m/z* 579), suggesting **26** is RES diglucuronide. Peak **27**, with a molecular ion at *m/z* 581 $[M - H]^-$ (229 + 176 × 2) which is 2 units higher than **26**, had a major MS/MS fragment at *m/z* 405 $[M - 176 - H]^-$ (loss of one glucuronide moiety from *m/z* 581), indicating **27** is DH-RES diglucuronide. In a similar manner, peak **28** was identified as RES sulfoglucuronide, **29** as DH-RES sulfoglucuronie, **30** as RES glucuronide, **31** as DH-RES glucuronide, and **32** as DH-RES sulfate. Peak **33** (Rt 34.3 min), having an identical MS/MS spectrum but a different retention time to that of **1** (Rt 30.4 min), was tentatively identified as *cis*-RES.[33] As a matter of fact, conjugates **25–32** have been previously identified as the phase II metabolites of RES in pigs after intragastric RES administration.[33]

As shown in Figure 4, RAH was found to be well tolerated in the acidic environment of stomach and only small amounts of DA-RAH **10** appeared in mouse stomach contents in 1 h after administration (Figure 4C). After 2 h, most RAH was transited out of the stomach (Figure 4D). In contrast, an increasing amount of RAH was found in mouse intestine contents from 1 h to 2 h after administration (Figures 4F and 4G). Interestingly, RAH was found to be relatively abundant in the mouse feces collected in 24 h after oral administration (Figure 4H). Particularly, intact RAH was also detected in the mouse colon tissue collected at 1 h and 2 h after

administration (Figures 4I and 4J). Our observations suggested that certain amount of RAH is able to bear the acidic environment of stomach, avoid enzymatic hydrolysis in the small intestine, reach the colon, and then incorporated into the colon tissue as an intact molecule, thereby contributing its potential chemopreventive activity in colon cancer, like RES reported previously.[34]

On the other hand, salicylic acid **23** and RES sulfate **25** were detected as the major metabolites of RAH in the mouse plasma collected at 2 h after administration (Figure 4K and 4L). The metabolites found in the colon tissue at 2 h were salicylic acid **23**, RES **1** and RES sulfate **25** (Figures 4I and 4J). In addition, four RES conjugates (sulfate **25**, diglucuronide **26**, sulfoglucuronide **28**, and glucuronide **30**), salicyluric acid **22**, and salicylic acid **23** were excreted into the mouse urine collected in 24 h after administration (Figure 4M). These observations indicated that the majority of RAH is capable of rapidly releasing RES and ASA or salicylic acid after incorporation into intestinal enterocyte. Whereas, the arise of RES **1**, salicyluric acid **22** and salicylic acid **23** in the intestine contents (Figures 4F and 4G) also suggested certain amount of RAH collapses in the intestine and subsequently releases RES and salicylic acid. The released RES and salicylic acid in the intestine may be reabsorbed into blood circulation later. This hypothesis is supported by the presence of microbiota-derived metabolite DH-RES **24** in the mouse feces (Figure 4H) and four DH-RES conjugates (diglucuronide **27**, sulfoglucuronide **29**, glucuronide **31**, and sulfate **32**) in the mouse urine (Figure 4M).[33] As a result, RAH is capable of releasing RES and ASA or salicylic acid for their respective biological effects either in the intestine or after absorption via intestinal enterocyte. Thus, ASA or its metabolite salicylic acid displays anti-inflammatory and anti-cancer properties while RES showing gastroprotective effects and cancer prevention. At this point, it is worth emphasizing that ASA is not able to be

directly detected in mice, probably due to its rapid hydrolysis to salicylic acid *in vivo*. The reported biologic half-life of ASA is very short, approximately 15 to 20 min in normal adults.[35] As a result, our study on the metabolic fate and distribution of RAH in RAH-treated mice demonstrated 1) RAH is well tolerated in the stomach; 2) RAH is capable of releasing RES and ASA or salicylic acid for their respective biological effects either in the intestine or after absorption via intestinal enterocyte; and 3) certain amount of RAH could reach the target sites of the colon and thereby contribute its enhanced chemoprevention activities as an intact molecule.

RAH Inhibits Human Colon Cancer Cell Cycle Arrest via Down-regulation of Cyclin D1 and Cyclin E. Cell proliferation is known to follow the orderly progression of the cell cycle, which is governed by protein complexes composed of cyclins such as cyclins D1 and E, and cyclin-dependent kinases.[36, 37] To determine whether the cytotoxic effects induced by the treatment of RAH are due to cell cycle arrest, we performed flow cytometry analysis. HCT-116 and HT-29 cells were treated with RAH at concentrations of 0, 20, 40, and 60 μM for the duration of 24 h, respectively. Our results showed that RAH could dose-dependently induce cell cycle arrest at the G1 phase in HCT-116 cells and G1 and S phases in HT-29 cells (Figure 5A). Further Western blot analysis showed that the expression of cyclins D1 and E in both cancer cells was significantly decreased in a dose-dependent manner after RAH treatment (Figure 5B). These results indicate that RAH can cause cell cycle arrest through down-regulation of cyclins D1 and E expression.

RAH Induces Cancer Cell Apoptosis through Mitochondrial Pathway by Activation of Caspase-3. We further investigated the potential of RAH to induce apoptosis in HCT-116 and HT-29 cells using flow cytometry (Figure 6A). We observed that RAH dose-dependently induced apoptosis in both HCT-116 and HT-29 cancer cells (Figure 6A). It is well documented

that mitochondria plays a key role in the apoptotic process,[38, 39] and pro-apoptotic members of the Bcl-2 family proteins can induce the release of cytochrome *c* into the cytosol which, once there, activates caspases by triggering mitochondrial membrane permeabilization, leading to cell death.[40-42] In order to determine how RAH induces apoptosis in colon cancer cells, we performed a Western blot analysis for Bcl-2 family proteins Bim, Bax, Bad, BclXL, Bcl2 and Bak, and cytochrome *c* as well as cleaved caspase-3 (Figures 6B and C). Our results showed that RAH up-regulated the expression of Bim, Bax, Bad, and Bak while decreasing the levels of Bcl2 and BclXL in both cell lines in a dose-dependent manner (Figure 6B). Increased expression of cytochrome *c* was clearly observed in both cell lines (Figure 6B). In addition, we also found the expression of cleaved caspase-3 increased significantly with increasing doses of RAH (Figure 6C). These observations indicate that RAH induces the release of cytochrome *c* into cytoplasm to activate downstream caspase-3, thereby leading to cell death in human colon cancer cells HCT-116 and HT-29.

CONCLUSION

In summary, a novel class of RES-based ASA prodrugs have been designed, synthesized, and characterized for the first time, and their anticancer properties have also been validated. We identified RAH **4** as the most potent anticancer agent, which is more active than a physical mixture of ASA and RES, individual ASA or individual RES. We further validated the antiproliferative properties of the molecular skeleton composed of ASA and RES by using stable synthetic RAH resemblances **11** and **12**. Stability of RAH in cancer cells revealed that RAH was hydrolyzed into DA-RAH and subsequently decomposed to release RES and salicylic acid over 24 h of incubation. RAH was further found to be well tolerated in the stomach in mice after intragastric RAH administration, far beyond the common sense of susceptibility of an ester in the

acidic environment. The majority of RAH is capable of releasing RES and ASA or salicylic acid for their respective biological effects either in the intestine or after absorption via intestinal enterocyte, while certain amount of RAH could reach the target sites of the colon and thereby contribute its enhanced chemoprevention activities as an intact molecule. Further mechanistic studies demonstrated RAH inhibits cancer cell cycle arrest through down-regulation of cyclin D1 and E, and induces cancer cell apoptosis through mitochondrial pathway by activation of caspase-3. As a combination of NSAIDs and dietary polyphenol, RAH represents a class of novel and safer alternatives of ASA, with the ability to release simultaneously RES and ASA, which effectively retain the broad spectrum of effects for ASA while reducing the side effect of gastrointestinal ulceration. RAH may be developed as a pharmaceutical for the purpose of chronic use in the prophylaxis and/or treatment of colon cancer and other diseases in clinical trials in the future.

EXPERIMENTAL SECTION

Chemistry. *General Methods.* Anhydrous reactions were carried out in oven-dried glassware under a nitrogen atmosphere unless otherwise noted. Reactions were monitored by analytical thin-layer chromatography (TLC) on 250 μm silica gel plates (GF254) (Merck) and visualized under UV light. The products were isolated and purified by either preparative TLC on 2000 μm silica gel plates (GF254) (Sorbent Technologies, catalog no. 1617124) or column chromatography (CC) using silica gel (Sorbent Technologies, catalog no. 3093M-25). ^{1}H, ^{13}C NMR, and two-dimensional (2-D) NMR spectra were recorded on a Bruker AVANCE 600 MHz or 700 MHz spectrometer (Brucker, Inc., Silberstreifen, Rheinstetten, Germany) using TMS as an internal standard. Chemical shifts (δ) are expressed in ppm. Coupling constants (J) are expressed in Hz, and multiplicities are indicated by s (singlet), d (doublet), t (triplet), q (quartet),

and br (broad). The ^{13}C NMR spectra are proton decoupled. Resveratrol was obtained from Mega Resveratrol (Danbury, CT, USA). Aspirin was purchased from MP Biomedicals (Solon, OH). Salicylic acid was bought from Avantor Performance Materials (Phinllipsburg, NJ). Salicyluric acid was obtained from Sigma (St. Louis, MO). Sulfatase and β-glucuronidase were acquired from Sigma (St. Louis, MO). Other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and were used without further purification. All synthesized compounds were >95% pure.

HPLC Analysis. An HPLC-CEAD system (ESA, Chelmsford, MA) consisting of an ESA model 584 HPLC pump, an ESA model 542 autosampler, an ESA organizer, and an ESA coularray detector coupled with two ESA model 6210 four sensor cells was used in the current study. A Gemini C18 column (150 mm × 4.6 mm, 5 μm; Phenomenex, Torrance, CA) was used to analyze at a flow rate of 1.0 mL/min. The mobile phases consisted of solvent A (30 mM sodium phosphate buffer containing 1.75% acetonitrile and 0.125% tetrahydrofuran, pH 3.35) and solvent B (15 mM sodium phosphate buffer containing 58.5% acetonitrile and 12.5% tetrahydrofuran, pH 3.45). The gradient elution had the following profile: 20% B from 0 to 3 min; 20-55% B from 3 to 11 min; 55-60% B from 11 to 12 min; 60-65% B from 12 to 13 min; 65-100% B from 13 to 40 min; 100% B from 40 to 45 min; and 20% B from 45.1 to 50 min. The cells were then cleaned at a potential of 1000 mV for 1 min. The injection volume of the sample was 10 μL. The eluent was monitored by the Coulochem electrode array system (CEAS) with potential settings at -100, 0, 100, 200, 300, 400, and 500 mV.

LC/MS Analysis. LC/MS analysis was carried out with a Thermo-Finnigan Spectra System which consisted of an Accela high speed MS pump, an Accela refrigerated autosampler, and an LCQ Fleet ion trap mass detector (Thermo Electron, San Jose, CA, USA) incorporated with an electrospray ionization (ESI) interface. A Kinetex C18 column (150 mm × 4.6 mm i.d., 2.6 μm,

Phenomenex) was used for separation at a flow rate of 0.3 mL/min. The column was eluted with 100% A (5% aqueous methanol with 0.2% acetic acid (AA) for 5 min, followed by linear increases in B (95% aqueous methanol with 0.2% AA) to 30% from 5 to 10 min, to 50% from 10 to 35 min, then to 100% B from 35 to 40 min, and then with 100% B from 40 to 45 min. The column was then re-equilibrated with 100% A for 5 min. The injection volume was 10 μL for each sample. The LC eluent was introduced into the ESI interface. The negative ion polarity mode was set for the ESI source with the voltage on the ESI interface maintained at approximately 3.0 kV. Nitrogen gas was used as the sheath gas and auxiliary gas. Optimized source parameters, including capillary temperature (297 ^{o}C), sheath gas flow rate (20 arbitrary units), auxiliary gas flow rate (10 units), and tube lens (-80 V), and capillary voltage -24 V, were tuned using RES in methanol (10 μg/mL). The collision-induced dissociation (CID) was conducted with an isolation width 2 Da and normalized collision energy of 35 for MS/MS. The mass range was measured from 100 to 800 *m/z*. Data acquisition was performed with Xcalibur version 2.0 (Thermo Electron, San Jose, CA, USA).

General Procedure A. To a solution of stilbenes (1.0 eq.) and Et_3N (3.0 eq.) in DCM at 0 ^{o}C was added a solution of *O*-acetylsalicyloyl chloride (1.0 eq.-2.0 eq.) in DCM dropwise. After addition, the mixture was stirred at 0 ^{o}C for 30 min and allowed to warm up to rt for another 2 h. The reaction was quenched with water and extracted with ethyl acetate (EA). The organic phase was washed with water (× 2) and brine (× 1), dried over Na_2SO_4, and filtered. The filtrate was evaporated *in vacuo*, and the residue was subjected to column chromatography (CC) to give the desired compounds.

General Procedure B. To a solution of methyl ether derivatives (1.0 eq.) in DCM at 0 ^{o}C or -78 ^{o}C was added a solution of BBr_3 in DCM (1.0 M, 2.0-3.0 eq.) dropwise. The mixture was

stirred at certain temperature for 0.5 h-3 h and quenched with water. The resulting mixture was extracted with EA. The organic solution was washed with water (× 2) and brine (× 1), dried over Na_2SO_4, and filtered. The filtrate was evaporated in vacuo, and the residue was purified by CC to give the desired compounds.

General Procedure C. A mixture of triphenylphosphine (TPP) (1.0 eq.) and halides (1.0 eq.) in toluene was refluxed under N_2 overnight. The mixture was cooled down to rt, and hexane was added. The solid was filtered and washed with hexane/EA (10:1) to give the corresponding alkylphosphoniums. To a stirred solution of 3,5-dimethoxybenzaldehyde (1.2 eq.) in THF was added alkylphosphoniums (1.0 eq.). The resulting mixture was then cooled down to 0 ^oC and $NaOBu^{-t}$ (1.2 eq.) was added. After addition, the mixture was allowed to warm up to rt and stirred for 6 h-18 h under N_2. The reaction was quenched by saturated NH_4Cl aqueous. The mixture was extracted with EA. The organic solution was washed with water (× 2) and brine (× 1), dried over Na_2SO_4, and filtered. The filtrate was evaporated in vacuo, and the residue was purified by CC to give the desired compounds.

General Procedure D. A mixture of bromide (1.0 eq.), 1,3-dimethoxy-5-vinylbenzene (1.2 eq.), $Pd(PPh_3)_4$ (0.05 eq.), and TEA (2.0 eq) in dry DMF was heated to 100 ^oC and stirred for 18 h-24 h under N_2. The dark mixture was distributed between EA and 10% HCl aqueous. The organic layer was separated and washed with water (× 2) and brine (× 1), dried over Na_2SO_4, and filtered. The filtrate was evaporated in vacuo, and the residue was purified by pre-TLC to give the desired compounds.

*(E)-4-(3,5-Dihydroxystyryl)phenyl 2-acetoxybenzoate (**4**) and (E)-3-(4-Hydroxystyryl)-5-hydroxyphenyl 2-acetoxybenzoate (**5**).* General procedure A was followed using RES **1** (57 mg, 0.25 mmol, 1.0 eq.), *O*-acetylsalicyloyl chloride (50 mg, 0.25 mmol, 1.0 eq.) and Et_3N (0.14 mL,

0.75 mmol, 3.0 eq.) in DCM (5 mL). The residue was purified by prep-TLC (C/M = 15:1, and 10:1) to afford **4** (28 mg, yield: 78%) as a white solid and **5** (10 mg, yield: 28%) as a white solid. **4**: ^{1}H NMR (600 MHz, CD_3OD) δ 6.50 (2H, d, *J* = 2.0 Hz, H-2/6), 6.21 (1H, t, *J* = 2.0 Hz, H-4), 6.99 (1H, d, *J* = 16.3 Hz, H-7), 7.06 (1H, d, *J* = 16.3 Hz, H-8), 7.58 (2H, d, *J* = 8.6 Hz, H-10/14), 7.15 (2H, d, *J* = 8.6 Hz, H-11/13), 7.22 (1H, d, *J* = 8.0 Hz, H-19), 7.69 (1H, dt, *J* = 8.0, 1.6 Hz, H-20), 7.43 (1H, t, *J* = 7.8 Hz, H-21), 8.18 (1H, dd, *J* = 7.8, 1.6 Hz, H-22), and 2.26 (3H, s, $C\underline{H}_3C{=}O$); ^{13}C NMR (150 MHz, CD_3OD) δ 140.6 (s, C-1), 106.2 (d, C-2/6), 159.8 (s, C-3/5), 103.3 (d, C-4), 130.5 (d, C-7), 128.3 (d, C-8), 137.0 (s, C-9), 128.5 (d, C-10/14), 123.0 (d, C-11/13), 151.3 (s, C-12), 164.6 (s, C-16), 124.0 (s, C-17), 152.4 (s, C-18), 125.1 (d, C-19), 135.9 (d, C-20), 127.4 (d, C-21), 133.0 (d, C-22), 171.3 (s, $CH_3\underline{C}{=}O$), and 21.0 (q, $\underline{C}H_3C{=}O$); negative ESIMS, *m/z* 449 $[M + CH_3COOH - H]^-$ and 389 $[M - H]^-$. **5**: ^{1}H NMR (600 MHz, CD_3OD) δ 6.80 (1H, brs, H-2), 6.47 (1H, t, *J* = 2.0 Hz, H-4), 6.86 (1H, brs, H-6), 6.89 (1H, d, *J* = 16.3 Hz, H-7), 7.05 (1H, d, *J* = 16.3 Hz, H-8), 7.38 (2H, d, *J* = 8.6 Hz, H-10/14), 6.76 (2H, d, *J* = 8.6 Hz, H-11/13), 7.23 (1H, d, *J* = 8.0 Hz, H-19), 7.70 (1H, dt, *J* = 8.0, 1.6 Hz, H-20), 7.44 (1H, t, *J* = 8.0 Hz, H-21), 8.17 (1H, dd, *J* = 8.0, 1.6 Hz, H-22), and 2.29 (3H, s, $C\underline{H}_3C{=}O$); ^{13}C NMR (150 MHz, CD_3OD) δ 141.9 (s, C-1), 111.5 (d, C-2), 153.2 (s, C-3), 108.6 (d, C-4), 159.8 (s, C-5), 111.8 (d, C-6), 125.8 (d, C-7), 130.8 (d, C-8), 130.1 (s, C-9), 129.1 (d, C-10/14), 116.5 (d, C-11/13), 158.7 (s, C-12), 164.6 (s, C-16), 124.1 (s, C-17), 152.4 (s, C-18), 125.1 (d, C-19), 135.9 (d, C-20), 127.4 (d, C-21), 133.0 (d, C-22), 171.4 (s, $CH_3\underline{C}{=}O$), and 21.3 (q, $\underline{C}H_3C{=}O$); negative ESIMS, *m/z* 449 $[M + CH_3COOH - H]^-$ and 389 $[M - H]^-$.

*(E)-4-(3,5-Dimethoxystyryl)phenyl 2-acetoxybenzoate (**6**).* General procedure A was followed using pterostilbene **2** (256 mg, 1.0 mmol, 1.0 eq.), *O*-acetylsalicyloyl chloride (397 mg, 2.0 mmol, 2.0 eq.) and Et_3N (0.42 mL, 3.0 mmol, 3.0 eq.) in DCM (10 mL). The residue was

purified by CC (H/E = 20:1, 10:1, and 6:1) to give **6** (400 mg, yield: 96%) as a white solid. ^{1}H NMR (700 MHz, $CDCl_3$) δ 6.66 (2H, d, *J* = 2.0 Hz, H-2/6), 6.39 (1H, t, *J* = 2.0 Hz, H-4), 6.99 (1H, d, *J* = 16.1 Hz, H-7), 7.07 (1H, d, *J* = 16.1 Hz, H-8), 7.54 (2H, d, *J* = 8.4 Hz, H-10/14), 7.16 (2H, d, *J* = 8.4 Hz, H-11/13), 7.17 (1H, m, H-19), 7.64 (1H, dt, *J* = 7.7, 1.4 Hz, H-20), 7.38 (1H, t, *J* = 7.7 Hz, H-21), 8.21 (1H, dd, *J* = 7.7, 1.4 Hz, H-22), 2.30 (3H, s, C<u>H</u>$_3$C=O), and 3.80 (6H, s, OMe-3/5); ^{13}C NMR (175 MHz, $CDCl_3$) δ 139.2 (s, C-1), 104.6 (d, C-2/6), 161.0 (s, C-3/5), 100.1 (d, C-4), 129.1 (d, C-7), 128.1 (d, C-8), 135.3 (s, C-9), 127.7 (d, C-10/14), 122.0 (d, C-11/13), 150.0 (s, C-12), 163.0 (s, C-16), 122.5 (s, C-17), 151.2 (s, C-18), 124.1 (d, C-19), 134.7 (d, C-20), 126.3 (d, C-21), 132.3 (d, C-22), 169.8 (s, CH_3<u>C</u>=O), 21.1 (q, <u>C</u>H_3C=O), and 55.4 (q, OMe-3/5); negative ESIMS, *m/z* 477 $[M + CH_3COOH - H]^-$ and 417 $[M - H]^-$.

(E)-4-(3,5-Dimethoxystyryl)phenyl 2-hydroxybenzoate (7). To a solution of (*E*)-4-(3,5-dimethoxystyryl)phenyl 2-acetoxybenzoate **6** (100 mg, 0.24 mmol, 1.0 eq.) in THF (5 mL) at rt was added a deluted HCl aq. solution (3.0 M, 5 mL). After addition, the mixture was stirred at rt for 24 h and then heated to 60 ^{o}C for another 6 h. The reaction was cooled back to rt and distributed between EA (10 mL) and water (20 mL). The organic solution was separated, washed with water (10 mL × 2) and brine (10 mL × 1), dried over Na_2SO_4, and filtered. The filtrate was evaporated in vacuo, and the residue was purified by CC (H/E = 10:1) to give **7** (74 mg, yield: 82%) as a yellow solid. ^{1}H NMR (600 MHz, $CDCl_3$) δ 6.67 (2H, d, *J* = 2.2 Hz, H-2/6), 6.41 (1H, t, *J* = 2.2 Hz, H-4), 7.02 (1H, d, *J* = 16.3 Hz, H-7), 7.09 (1H, d, *J* = 16.3 Hz, H-8), 7.57 (2H, d, *J* = 8.6 Hz, H-10/14), 7.21 (2H, d, *J* = 8.6 Hz, H-11/13), 7.04 (1H, d, *J* = 8.6 Hz, H-19), 7.54 (1H, dt, *J* = 8.6, 1.6 Hz, H-20), 6.97 (1H, t, *J* = 8.0 Hz, H-21), 8.07 (1H, dd, *J* = 8.0, 1.6 Hz, H-22), 3.83 (6H, s, OMe-3/5), and 10.48 (1H, s, OH-18); ^{13}C NMR (150 MHz, $CDCl_3$) δ 139.1 (s, C-1), 104.7 (d, C-2/6), 161.0 (s, C-3/5), 100.2 (d, C-4), 129.3 (d, C-7), 128.0 (d, C-8), 135.5 (s, C-9),

127.6 (d, C-10/14), 121.9 (d, C-11/13), 149.5 (s, C-12), 168.9 (s, C-16), 111.8 (s, C-17), 162.2 (s, C-18), 117.9 (d, C-19), 136.5 (d, C-20), 119.5 (d, C-21), 130.4 (d, C-22), and 55.4 (q, OMe-3/5); negative ESIMS, *m/z* 435 $[M + CH_3COOH - H]^-$ and 375 $[M - H]^-$.

*(E)-4-(3-Hydroxy-5-methoxystyryl)phenyl 2-acetoxybenzoate (**8**).* General procedure B was followed using (*E*)-4-(3,5-dimethoxystyryl)phenyl 2-acetoxybenzoate **6** (209 mg, 0.5 mmol, 1.0 eq.) and BBr_3 (1.0 mmol, 2.0 eq.) in DCM (10 mL) at -78 ^oC. The mixture was stirred at -78 ^oC for 1.0 h. The residue was subjected to CC (H/E = 5:1, 4:1, 3:1, and 2:1) and further purified by pre-TLC (H/E = 3:1) to yield **8** (6.0 mg, yield: 3%) as a white solid. ^1H NMR (600 MHz, $CDCl_3$) δ 6.63 (1H, brs, H-2), 6.33 (1H, t, *J* = 2.2 Hz, H-4), 6.58 (1H, brs, H-6), 6.95 (1H, d, *J* = 16.3 Hz, H-7), 7.05 (1H, d, *J* = 16.3 Hz, H-8), 7.52 (2H, d, *J* = 8.5 Hz, H-10/14), 7.16 (2H, d, *J* = 8.5 Hz, H-11/13), 7.17 (1H, d, *J* = 8.0 Hz, H-19), 7.64 (1H, dt, *J* = 8.0, 1.5 Hz, H-20), 7.39 (1H, t, *J* = 8.0 Hz, H-21), 8.22 (1H, dd, *J* = 8.0, 1.6 Hz, H-22), 2.31 (3H, s, $C\underline{H}_3C{=}O$), and 3.81 (3H, s, OMe-3); ^{13}C NMR (150 MHz, $CDCl_3$) δ 139.5 (s, C-1), 105.0 (d, C-2), 161.2 (s, C-3), 101.1 (d, C-4), 156.8 (s, C-5), 105.9 (d, C-6), 128.7 (d, C-7), 128.3 (d, C-8), 136.2 (s, C-9), 127.7 (d, C-10/14), 124.1 (d, C-11/13), 150.0 (s, C-12), 163.0 (s, C-16), 123.1 (s, C-17), 151.5 (s, C-18), 122.0 (d, C-19), 134.7 (d, C-20), 126.2 (d, C-21), 132.2 (d, C-22), 55.4 (q, OMe-3), 169.8 (s, $CH_3\underline{C}{=}O$), and 21.0 (q, $\underline{C}H_3C{=}O$); negative ESIMS, *m/z* 463 $[M + CH_3COOH - H]^-$ and 403 $[M - H]^-$.

*(E)-4-(3-Hydroxy-5-methoxystyryl)phenyl 2-hydroxybenzoate (**9**) and (E)-4-(3,5-Dihydroxystyryl)phenyl 2-hydroxybenzoate (**10**).* General procedure B was followed using (*E*)-4-(3,5-dimethoxystyryl)phenyl 2-acetoxybenzoate **6** (400 mg, 0.96 mmol, 1.0 eq.) and BBr_3 (1.91 mmol, 2.0 eq.) in DCM (10 mL) at 0 ^oC. The mixture was stirred at 0 ^oC for 30 min. The residue was purified by CC (H/E = 10:1, 6:1, and 5:1; and then C/M = 50:1) to give **9** (110 mg, yield:

31%) as a white solid and **10** (65 mg, yield: 20%) as a white solid. **9**: ^{1}H NMR (600 MHz, $CDCl_3$) δ 6.64 (1H, brs, H-2), 6.35 (1H, t, *J* = 2.0 Hz, H-4), 6.60 (1H, brs, H-6), 6.96 (1H, d, *J* = 16.3 Hz, H-7), 7.06 (1H, d, *J* = 16.3 Hz, H-8), 7.55 (2H, d, *J* = 8.5 Hz, H-10/14), 7.20 (2H, d, *J* = 8.5 Hz, H-11/13), 7.04 (1H, d, *J* = 8.0 Hz, H-19), 7.54 (1H, m, H-20), 6.97 (1H, m, H-21), 8.07 (1H, dd, *J* = 8.0, 1.5 Hz, H-22), 3.81 (3H, s, OMe-3), and 10.48 (1H, s, OH-18); ^{13}C NMR (125 MHz, $CDCl_3$) δ 139.4 (s, C-1), 105.1 (d, C-2), 161.2 (s, C-3), 101.2 (d, C-4), 156.8 (s, C-5), 106.0 (d, C-6), 129.0 (d, C-7), 128.2 (d, C-8), 135.5 (s, C-9), 127.7 (d, C-10/14), 121.9 (d, C-11/13), 149.5 (s, C-12), 168.9 (s, C-16), 111.8 (s, C-17), 162.2 (s, C-18), 117.9 (d, C-19), 136.6 (d, C-20), 119.5 (d, C-21), 130.4 (d, C-22), and 55.4 (q, OMe-3); negative ESIMS, *m/z* 421 $[M + CH_3COOH - H]^-$ and 361 $[M - H]^-$. **10**: ^{1}H NMR (600 MHz, CD_3OD) δ 6.51 (2H, d, *J* = 2.0 Hz, H-2/6), 6.21 (1H, t, *J* = 2.0 Hz, H-4), 6.99 (1H, d, *J* = 16.3 Hz, H-7), 7.07 (1H, d, *J* = 16.3 Hz, H-8), 7.60 (2H, d, *J* = 8.5 Hz, H-10/14), 7.22 (2H, d, *J* = 8.5 Hz, H-11/13), 7.02 (1H, m, H-19), 7.56 (1H, dt, *J* = 7.8, 1.6 Hz, H-20), 7.00 (1H, m, H-21), and 8.07 (1H, dd, *J* = 7.9, 1.4 Hz, H-22); ^{13}C NMR (125 MHz, CD_3OD) δ 140.6 (s, C-1), 106.2 (d, C-2/6), 159.8 (s, C-3/5), 103.4 (d, C-4), 130.6 (d, C-7), 128.3 (d, C-8), 137.2 (s, C-9), 128.5 (d, C-10/14), 123.1 (d, C-11/13), 151.0 (s, C-12), 170.0 (s, C-16), 113.2 (s, C-17), 163.3 (s, C-18), 118.7 (d, C-19), 137.6 (d, C-20), 120.7 (d, C-21), and 131.5 (d, C-22); negative ESIMS, *m/z* 407 $[M + CH_3COOH - H]^-$ and 347 $[M - H]^-$.

*(E)-1-(3,5-Dimethoxystyryl)-4-bromobenzene (**14**).* General procedure C was followed using (4-bromobenzyl)triphenylphosphonium bromide (2.2 g, 4.3 mmol, 1.0 eq.) [Prepared from 4-bromobenzyl bromide **13** (1.24 g, 5.0 mmol, 1.0 eq.) and PPh_3 (1.31 g, 5.5 mmol, 1.1 eq.) in toluene (10 mL)], 3,5-dimethoxybenzaldehyde (863 mg, 5.2 mmol, 1.2 eq.) and $NaOBu^{-t}$ (499 mg, 5.2 mmol, 1.2 eq.) in THF (20 mL). The mixture was stirred at rt for 6 h. The residue was

purified by CC (H/E = 60:1 and 40:1) to give **14** (0.9 g, yield: 66%) as a white solid. ^{1}H NMR (700 MHz, $CDCl_3$) δ 6.65 (2H, d, *J* = 2.2 Hz, H-2/6), 6.40 (1H, t, *J* = 2.2 Hz, H-4), 7.02 (1H, d, *J* = 16.2 Hz, H-7), 6.99 (1H, d, *J* = 16.2 Hz, H-8), 7.47 (2H, d, *J* = 8.5 Hz, H-10/14), 7.36 (2H, d, *J* = 8.5 Hz, H-11/13), and 3.82 (6H, s, OMe-1/3); negative ESIMS, *m/z* 317 $[M - H]^-$, and 319 $[M + 2 - H]^-$.

*(E)-2-((4-(3,5-Dimethoxystyryl)phenyl)(hydroxy)methyl)phenol (**15**).* To a solution of (*E*)-1-(4-bromostyryl)-3,5-dimethoxybenzene **14** (700 mg, 2.20 mmol, 1.5 eq.) in dry THF (5 mL) at -78 °C was added n-BuLi (1.06 mL, 2.5 M in hexane, 2.64 mmol, 1.8 eq.). One hour after the addition, salicylaldehyde (179 mg, 1.47 mmol, 1.0 eq.) in THF (2 mL) was added. The reaction mixture was stirred at -78 °C for another 2 h and water (10 mL) was added to quench the reaction. The resulting mixture was extracted with EA (10 mL × 3). The extracts were combined and dried over Na_2SO_4. Evaporation of solvent followed by CC (H/E = 20:1, 10:1, 5:1 and 3:1) to afford **15** (449 mg, yield: 66%) as yellow oil. ^{1}H NMR (700 MHz, $CDCl_3$) δ 6.34 (2H, d, *J* = 2.2 Hz, H-2/6), 6.29 (1H, t, *J* = 2.2 Hz, H-4), 6.68-6.62 (2H, m, H-7/8), 7.48 (2H, d, *J* = 8.3 Hz, H-10/14), 7.36 (2H, d, *J* = 8.3 Hz, H-11/13), 5.95 (1H, s, H-15), 6.79-6.76 (1H, m, H-18), 7.20-7.15 (1H, m, H-19), 6.88-6.83 (1H, m, H-20), 7.06-7.02 (1H, m, H-21), and 3.81 (6H, s, OMe-1/3); negative ESIMS, *m/z* 361 $[M - H]^-$.

*(E)-(4-(3,5-Dimethoxystyryl)phenyl)(2-hydroxyphenyl)methanone (**16**).* A mixture of (*E*)-2-((4-(3,5-dimethoxystyryl)phenyl)(hydroxy)methyl)phenol **15** (228 mg, 0.63 mmol, 1.0 eq.), $CuCl_2 \cdot 2H_2O$ (11 mg, 0.063 mmol, 0.1 eq) and K_2CO_3 (261 mg, 1.89 mmol, 3.0 eq.) in DMF (8 mL) was stirred at 60 °C for 18 h under air atmosphere. The mixture was neutralized to pH ~7.0 by adding aq. 10% HCl solution and extracted with EA (10 mL × 3). The organic extracts were washed with water (10 mL × 2) and brine (10 mL × 1), dried over Na_2SO_4, and filtered. The

filtrate was removed under reduced pressure, and the residue was purified by CC (H/E = 20:1 and 10:1) to give **16** (138 mg, yield: 61%) as a yellow solid. ^{1}H NMR (600 MHz, $CDCl_3$) δ 6.71 (2H, d, *J* = 2.2 Hz, H-2/6), 6.45 (1H, t, *J* = 2.2 Hz, H-4), 7.18 (1H, d, *J* = 16.3 Hz, H-7), 7.14 (1H, d, *J* = 16.3 Hz, H-8), 7.69 (2H, d, *J* = 8.2 Hz, H-10/14), 7.63 (2H, d, *J* = 8.2 Hz, H-11/13), 7.07 (1H, d, *J* = 8.3 Hz, H-18), 7.53-7.48 (1H, m, H-19), 6.86 (1H, t, *J* = 8.0 Hz, H-20), 7.64 (1H, d, *J* = 8.0 Hz, H-21), and 3.85 (6H, s, OMe-1/3); negative ESIMS, *m/z* 359 $[M - H]^-$.

*(E)-(4-(3,5-Dihydroxystyryl)phenyl)(2-hydroxyphenyl)methanone (**11**).* General procedure B was followed using (*E*)-(4-(3,5-dimethoxystyryl)phenyl)(2-hydroxyphenyl)methanone **16** (138 mg, 0.38 mmol, 1.0 eq.) and BBr_3 (1.34 mmol, 2.0 eq.) in DCM (10 mL). The mixture was stirred at -78 ^{o}C for 1 h and then allowed to warm up to rt for another 2 h. The residue was subjected to pre-TLC (C/M = 10:1) and further purified by LH-20 (EtOH) to give **11** (79 mg, yield: 62%) as a yellow solid. ^{1}H NMR (600 MHz, CD_3OD) δ 6.53 (2H, d, *J* = 2.0 Hz, H-2/6), 6.23 (1H, t, *J* = 2.0 Hz, H-4), 7.18 (1H, d, *J* = 16.3 Hz, H-7), 7.13 (1H, d, *J* = 16.3 Hz, H-8), 7.66 (2H, d, *J* = 8.3 Hz, H-10/14), 7.70 (2H, d, *J* = 8.3 Hz, H-11/13), 7.01 (1H, d, *J* = 8.3 Hz, H-18), 7.51 (1H, dt, *J* = 8.3, 1.5 Hz, H-19), 6.93 (1H, t, *J* = 8.0 Hz, H-20), and 7.58 (1H, dd, *J* = 8.0, 1.4 Hz, H-21); ^{13}C NMR (150 MHz, CD_3OD) δ 140.2 (s, C-1), 106.4 (d, C-2/6), 159.9 (s, C-3/5), 103.9 (d, C-4), 133.0 (d, C-7), 128.2 (d, C-8), 143.1 (s, C-9), 127.3 (d, C-10/14), 131.1 (d, C-11/13), 137.8 (s, C-12), 201.7 (t, C-15), 121.7 (s, C-16), 163.0 (s, C-17), 118.9 (d, C-18), 136.7 (d, C-19), 120.0 (d, C-20), and 133.9 (d, C-21); negative ESIMS, *m/z* 331 $[M - H]^-$.

*1,3-Dimethoxy-5-vinylbenzene (**18**).* General procedure C was followed using methyltriphenylphosphonium iodide (4.3 g, 10.6 mmol, 1.0 eq.) [Prepared from triphenylphosphine (2.62 g, 10.0 mmol) and iodomethane (1.42 g, 10.0 mmol) in toluene (20 mL)], 3,5-dimethoxybenzaldehyde **17** (2.1 g, 12.8 mmol, 1.2 eq.) and $NaOBu^{-t}$ (1.2 g, 12.8

mmol, 1.2 eq.) in THF (20 mL). The mixture was stirred at rt for 18 h. The residue was purified by CC (H/E = 60:1, 40:1, and 20:1) to give **18** (1.58 g, yield: 75%) as a colorless oil. ^{1}H NMR (600 MHz, $CDCl_3$) δ 6.57 (2H, d, *J* = 2.2 Hz, H-2/6), 6.39 (1H, t, *J* = 2.2 Hz, H-4), 6.65 (1H, dd, *J* = 17.5, 10.8 Hz, H-7), 5.74 (1H, d, *J* = 17.5 Hz, H-8a), 5.25 (1H, d, *J* = 10.8 Hz, H-8b), and 3.81 (6H, s); ^{13}C NMR (150 MHz, $CDCl_3$) δ 160.9 (s, C-1/3), 100.1 (d, C-2), 104.3 (d, C-4/6), 139.6 (s, C-5), 136.8 (d, C-7), 114.3 (t, C-8), and 55.3 (q, OMe-1/3); negative ESIMS, *m/z* 163 $[M - H]^-$.

*2-(2-(4-Bromophenyl)acetyl)phenyl acetate (**19**).* To a suspension of $Pd(PPh_3)_4$ (578 mg, 0.5 mmol, 0.05 eq.) and zinc powder (1.3 g, 20 mmol, 2.0 eq.) in dry THF (50 mL) was added 4-bromobenzyl bromide **13** (2.48 g, 10 mmol, 1.0 eq.) and *O*-acetylsalicyloyl chloride (2.08 g, 10.5 mmol, 1.05 eq.) at rt under N_2. The mixture was stirred at rt for 4 h and filtered. The filtration was concentrated under reduced pressure, and the residue was subjected to CC (H/E = 20:1, 10:1, and 5:1) to give **19** (1.0 g, yield: 30%) as a white solid. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.45 (2H, d, *J* = 8.4 Hz, H-3/5), 7.09 (2H, d, *J* = 8.4 Hz, H-2/6), 4.16 (2H, s, H-7), 7.13 (1H, dd, *J* = 8.0, 1.5 Hz, H-11), 7.54 (1H, dt, *J* = 8.0, 1.5 Hz, H-12), 7.32 (1H, dt, *J* = 8.0, 1.5 Hz, H-13), 7.79 (1H, dd, *J* = 8.0, 1.5 Hz, H-14), and 2.35 (3H, s, $C\underline{H_3}CO$); ^{13}C NMR (150 MHz, $CDCl_3$) δ 133.0 (s, C-1), 131.4 (d, C-2/6), 131.7 (d, C-3/5), 121.1 (s, C-4), 47.2 (t, C-7), 196.9 (s, C-8), 130.5 (s, C-9), 149.0 (s, C-10), 124.0 (d, C-11), 133.5 (d, C-12), 126.1 (d, C-13), 129.8 (d, C-14), 169.5 (s, $CH_3\underline{C}O$), and 21.1 (q, $\underline{C}H_3CO$); negative ESIMS, *m/z* 331 $[M - H]^-$ and 333 $[M + 2 - H]^-$.

*2-(4-Bromophenyl)-1-(2-hydroxyphenyl)ethanone (**20**).* General procedure B was followed using 2-(2-(4-bromophenyl)acetyl)phenyl acetate **19** (200 mg, 0.6 mmol, 1.0 eq.) and BBr_3 (0.89 mmol, 2.0 eq.) in DCM (5 mL). The mixture was stirred at -78 ^{o}C for 15 min. The residue was purified by pre-TLC (H/E = 10:1) to give **20** (168 mg, yield: 96%) as a white solid. ^{1}H NMR

(600 MHz, $CDCl_3$) δ 7.47 (2H, d, *J* = 8.3 Hz, H-3/5), 7.14 (2H, d, *J* = 8.3 Hz, H-2/6), 4.26 (2H, s, H-7), 6.99 (1H, d, *J* = 8.0 Hz, H-11), 7.48 (1H, t, *J* = 8.0 Hz, H-12), 6.91 (1H, t, *J* = 8.0 Hz, H-13), 7.82 (1H, d, *J* = 8.0 Hz, H-14), and 12.10 (1H, s, OH-10); ^{13}C NMR (150 MHz, $CDCl_3$) δ 132.8 (s, C-1), 131.2 (d, C-2/6), 131.9 (d, C-3/5), 118.9 (s, C-4), 44.4 (t, C-7), 203.2 (s, C-8), 121.3 (s, C-9), 162.9 (s, C-10), 118.8 (d, C-11), 136.8 (d, C-12), 119.1 (d, C-13), and 130.2 (d, C-14); negative ESIMS, *m/z* 289 $[M - H]^-$ and 291 $[M + 2 - H]^-$.

*2-(4-(3,5-Dimethoxystyryl)phenyl)-1-(2-hydroxyphenyl)ethanone (**21**).* General procedure D was followed using 2-(4-bromophenyl)-1-(2-hydroxyphenyl)ethanone **20** (168 mg, 0.58 mmol, 1.0 eq.), 1,3-dimethoxy-5-vinylbenzene **18** (114 mg, 0.7 mmol, 1.2 eq.), TEA (117 mg, 1.16 mmol, 2.0 eq.), and $Pd(PPh_3)_4$ (34 mg, 0.029 mmol, 0.05 eq.) in dry DMF (3 mL). The mixture was heated to 100 ^{o}C and stirred for 18 h. The residue was purified by pre-TLC (H/E = 10:1) to give **21** (32 mg, yield: 15%) as a white solid. ^{1}H NMR (600 MHz, $CDCl_3$) δ 6.65 (1H, d, *J* = 2.1 Hz, H-2/6), 6.39 (1H, t, *J* = 2.1 Hz, H-4), 7.00 (1H, d, *J* = 15.8 Hz, H-7), 7.05 (1H, d, *J* = 15.8 Hz, H-8), 7.48 (2H, d, *J* = 8.0 Hz, H-10/14), 7.25 (2H, d, *J* = 8.0 Hz, H-11/13), 4.30 (2H, s, H-15), 6.98 (1H, d, *J* = 8.0 Hz, H-19), 7.52 (1H, m, H-20), 6.90 (1H, t, *J* = 8.0 Hz, H-21), 7.86 (1H, d, *J* = 8.0 Hz, H-22), 3.82 (6H, s, OMe-1/3), and 12.19 (1H, s, OH-18); ^{13}C NMR (150 MHz, $CDCl_3$) δ 203.7, 162.9, 161.0, 139.3, 136.6, 136.2, 133.4, 130.4, 129.8, 129.7, 128.9, 128.6, 127.3, 127.2, 127.0, 119.0, 118.7, 55.4, and 44.9; negative ESIMS, *m/z* 373 $[M - H]^-$.

*2-(4-(3,5-Dihydroxystyryl)phenyl)-1-(2-hydroxyphenyl)ethanone (**12**).* General procedure B was followed using (*E*)-2-(4-(3,5-dimethoxystyryl)phenyl)-1-(2-hydroxyphenyl)ethanone **21** (32 mg, 0.086 mmol, 1.0 eq.) and BBr_3 (0.34 mmol, 2.0 eq.) in DCM (5 mL). The mixture was stirred at 0 ^{o}C for 1 h and then allowed to warm up to rt for another 2 h. The residue was subjected to pre-TLC (C/M = 10:1) and further purified by LH-20 (EtOH) to give **12** (20 mg,

yield: 67%) as a white solid. ^{1}H NMR (600 MHz, CD_3OD) δ 6.47 (2H, d, J = 2.0 Hz, H-2/6), 6.19 (1H, t, J = 2.0 Hz, H-4), 6.98 (1H, d, J = 16.3 Hz, H-7), 7.02 (1H, d, J = 16.3 Hz, H-8), 7.47 (2H, d, J = 8.1 Hz, H-10/14), 7.27 (2H, d, J = 8.1 Hz, H-11/13), 4.36 (2H, s, H-15), 6.93 (1H, t, J = 8.3 Hz, H-19), 7.49 (1H, m, H-20), 6.95 (1H, d, J = 8.3 Hz, H-21), and 8.02 (1H, dd, J = 8.3, 1.3 Hz, H-22); ^{13}C NMR (150 MHz, CD_3OD) δ 140.8 (s, C-1), 106.1 (d, C-2/6), 159.8 (s, C-3/5), 103.2 (d, C-4), 130.0 (d, C-7), 129.0 (d, C-8), 137.6 (s, C-9), 127.7 (d, C-10/14), 130.9 (d, C-11/13), 135.3 (s, C-12), 45.9 (t, C-15), 205.9 (s, C-16), 120.5 (s, C-17), 163.8 (s, C-18), 119.1 (d, C-19), 137.5 (d, C-20), 120.2 (d, C-21), and 132.2 (d, C-22); negative ESIMS, *m/z* 345 $[M - H]^-$.

Biological Assays. *Materials and Methods.* Human colon cancer cells HCT-116 and HT-29 were obtained from American Type Tissue Culture (Manassas, VA). McCoy's 5A medium was purchased from Thermo Fisher Scientific (Waltham, MA). Supplements of Fetal Bovine Serum (FBS) and penicillin/streptomycin were purchased from Gemini Bio-Products (West Sacramento, CA). Crystal violet, glutaraldehyde and propidium iodide were obtained from Thermo Fisher Scientific (Waltham, MA). Primary antibodies against Bim, Bax, Bid, Bad, Bak, BclXL, and Bcl-2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Cytochrome *c*, Cleaved Caspase 3, *β*-actin, as well as secondary antibodies conjugated to HRP (Horseradish Peroxydase) against mouse and rabbit were purchased from Cell Signaling Technology (Beverly, MA).

Metabolism of RAH in Human Colon Cancer Cells. Cancer cells HCT-116 (1.0×10^6) were plated in 6-well culture plates and allowed to attach for 24 h at 37 ^{o}C in a 5% CO_2 incubator. RAH in DMSO was added to McCoy's 5A medium (containing 10% fetal bovine serum, 1% penicillin/streptomycin, and 1% glutamine) to reach a final concentration of 20 μM and then incubated. At different time pints (0, 0.5, 1, 2, 4, 8, and 24 h), 100 μL samples of

supernatant were taken and transferred to vials. An equal volume of acetonitrile was added to the samples before centrifugation. The supernatant was harvested, and the samples were analyzed by HPLC-CEAD.

MTT Assay. Human colon cancer cells HCT-116 and HT-29 were plated in 96-well microtiter plates with 3000 cells/well and allowed to attach for 24 h at 37 ºC. The test compounds (in DMSO) were added to cell culture medium to the desired final concentrations (final DMSO concentrations for control and treatments were 0.1 %). After the cells were cultured for 24 h, the medium was aspirated and cells were treated with 200 μL fresh medium containing 2.41 mmol/L 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumomide (MTT). After incubation for 3 h at 37 ºC, the medium containing MTT was aspirated, 100 μL of DMSO was added to solubilize the formazan precipitate, and plates were shaken gently for 1 h at room temperature. Absorbance values were derived from the plate reading at 550 nm on a Biotek microtiter plate reader (Winooski, VT). The reading reflected the number of viable cells and was expressed as a percentage of viable cells in the control. Both HCT-116 and HT-29 cells were cultured in McCoy's 5A medium. The media used above was supplemented with 10 % fetal bovine serum, 1 % penicillin/streptomycin, and 1 % glutamine, and the cells were kept in a 37 ºC incubator with 95% humidity and 5% CO_2. The IC_{50} values were conducted by using GraphPad Prism software (version 5).

Colony Formation Assay. Human colon cancer cells HCT-116 or HT-29 (1,000 cells per well) were seeded in 6-well culture plates for 24 h and then incubated with the compounds in DMSO in a 37 °C incubator with 5% CO_2. After 2 weeks, colonies were washed with phosphate-buffered saline (PBS), then stained with a mixture of 6.0% glutaradehyde and 0.5% crystal violet for 30 min at room temperature, rinsed in water, air-dried, and then photographed.

Apoptosis and Cell Cycle Analysis. Apoptosis and cell cycle were determined by FACS analysis of propidium iodide (PI)-stained cells. In brief, cells were trypsinized, washed with cold phosphate-buffered saline (PBS), fixed in ice-cold 70% ethanol for 1 h, and then resuspended in 2 mL PBS supplemented with 10 μL RNase (100 mg/ml) and incubated at 37°C for 30 min. After incubation, DNA was stained with 1 mg/mL PI in PBS. Cell staining was analyzed using a Cell Lab QuantaTM SC flow cytometer (Beckman Coulter, Danvers, MA) and data were processed using FlowJo vesion 7.6.1 software (Tree Star, San Carlos, CA). The percentage of cell cycle distribution in each sample was determined based on the G_1, S and G_2 peaks detected in Watson model. The percentage of apoptotic cells in each sample was determined based on the sub G_1 peaks detected in monoparametric histograms.

Western Blot Analysis. Cell lysates were prepared in ice-cold RIPA lysis buffer [25 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, Thermo Fisher Scientific] supplemented with a protease inhibitor cocktail (AEBSF, aprotinin, bestatin, E-64, leupeptin and pepstatin A in DMSO with EDTA, Thermo Fisher Scientific). Protein content was measured by a Pierce BCA Assay Kit (Thermo Fisher Scientific). Protein contents of cell lysates (30 μg protein/lane) were resolved by SDS-PAGE. Proteins were then electro-transferred onto PVDF membranes and blots were blocked for one hour at room temperature in 1 × TBS with 1% Casein (Bio-Rad Laboratories, Berkeley, CA). Blots were then incubated overnight at 4 °C with the desired primary antibody diluted in TBS with 0.5% Tween-20. Blots were then washed with TBS-Tween 20 and probed for 1 h with the appropriate secondary antibody (1:1000). Protein bands were visualized with chemiluminescence using West Femto maximum detection substrate (Thermo Fisher Scientific). To confirm equal protein loading in each lane,

immunoblots were stripped and re-probed for β-actin. Protein fold-induction was calculated by normalizing the intensity of the band of interest to β-actin first, and then to control lanes.

Animals Study. *Animals and Study Design.* Experiments with mice were carried out according to protocols approved by the Institutional Review Board for the Animal Care and Facilities Committee at North Carolina Central University. Female C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and allowed to acclimate for at least 1 week prior to the start of the experiment. The mice were housed 5 per cage and maintained in air-conditioned quarters with a room temperature of 20 ± 2 ºC, relative humidity of 50 ± 10 %, and an alternating 12-h light/dark cycle. Mice were fed Rodent Chow #5001 (LabDiet) and water, and were allowed to eat and drink *ad libitum*. RAH in DMSO was administered to mice by oral gavage (200 mg/kg). Stomach contents, intestine contents, and colon tissue were collected at 1 h and 2 h, respectively, after administration of vehicle (control group, n = 5), and RAH (treated group, n = 5). Urine and fecal samples were collected in metabolism cages (5 mice per cage) in 24 h after administration. Blood samples were collected by cardiac puncture and plasma was obtained after centrifugation at 17,000 × g for 5 min at 4 ^{o}C. The samples were stored at -80 ºC until analysis.

Sample Preparation. For the metabolic profile, mouse urine samples (50 μL for each group) were added to 950 μL methanol to precipitate proteins. After centrifugation at 17,000 × g for 5 min, the supernatant was transferred into vials for LC/MS analysis. Enzymatic deconjugation of mouse urine was performed as described previously with slight modification.[43] In brief, triplicate samples were prepared in the presence of β-glucuronidase (250 U) and sulfatase (3 U) for 24 h at 37 ^{o}C. After incubation, the medium was diluted 5 times by adding methanol containing 0.2% AA. The resulting solution was centrifuged at 17,000 g for 5 min, and 10 μL of supernatant was

analyzed directly by LC/MS. Fecal samples (4 pieces) were selected and placed in 1.0 mL of MeOH (0.2% AA). Samples were homogenized for 5 min by an Omni Bead Ruptor Homogenizer (Kennesaw, GA) and then centrifuged at 17,000 g for 10 min. The supernatant (250 μL) was collected and diluted 5 times for analysis. Stomach contents or small intestine contents (5 mg), along with 500 μL methanol was sonicated until homogenous. Next the samples were centrifuged at 17,000 g for 10 min at 4 ^{o}C. Supernatant was filtered thru 0.22 μM filter and stored in vial for analysis. Colon tissues in 750 μL 60% methanol (1% AA) were placed in -20 ^{o}C for 10 min. Next, samples were homogenized for 5 min and then centrifuged at 4 ^{o}C for 10 min. 600 μL of supernatant was removed and dried out by vacufuge. Samples were redissolved in 200 μL 80% methanol (0.2% AA) and transferred to vials for analysis. Plasma samples (50 μL) were added to 950 μL methanol to precipitate proteins. After centrifugation at 17,000 × g for 5 min, the supernatant was transferred into vials for LC/MS analysis.

ASSOCIATED CONTENT

Supporting Information

NMR spectra (^{1}H NMR, ^{13}C NMR and 2-D NMR).

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

AA, acetic acid; ASA, aspirin; ASA+RES, an equimolar mixture of aspirin and resveratrol; CC, column chromatography; CEAD**,** coulometric electrode array detector; COX, cyclooxygenase; CRC, colorectal cancer; DH-RES, dehydroresveratrol; EA, ethyl acetate; GIT, gastrointestinal tract; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumomide; NO, nitric oxide; NSAIDs, nonsteroidal anti-inflammatory drugs; PGs, prostaglandins; RAH, resveratrol–aspirin hybrid; RES, resveratrol; TPP, triphenylphosphine.

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Table 1. The major RAH metabolites detected in RAH-treated mice (200 mg/kg, intragastric gavage)

No.	Metabolite[a]	Rt (min)	$[M - H]^-$	MS/MS	Occurrence[b]
1	RES	30.4	227	**227**/185	IC; Feces; Colon
5	RAH	43.0	389 449*	**389**/347, 303, 227 (B)	SC; IC; Feces; Colon
10	DA-RAH	43.9	347 407*	**347**/303, 227 (B)	SC; IC; Feces; Colon
22	Salicyluric acid	18.6	194	**194**/150	IC; Urine
23	Salicylic acid	23.9	137	**137**/93	IC; Feces; Colon; Plasma; Urine
24	DH-RES	30.4	229	**229**/187 (B), 161	Feces; Urine
25	RES sulfate	26.8	307	**307**/243, 227 [M – sulfate – H]$^-$ (B)	Colon; Plasma; Urine
26	RES diglucuronide	16.9	579	**579**/403 [M – glucuronide – H]$^-$	Urine
27	DH-RES diglucuronide	17.8	581	**581**/405 [M – glucuronide – H]$^-$	Urine
28	RES sulfoglucuronide	19.5	483	**483**/307 [M – glucuronide – H]$^-$	Urine
29	DH-RES	19.4	485	**485**/309 [M – glucuronide –	Urine

	sulfoglucuronide			$H]^-$	
30	RES glucuronide	22.2	403	**403**/385, 227 [M – glucuronide – $H]^-$, 175 (B)	Urine
31	DH-RES glucuronide	23.4	405	**405**/387, 229 [M – glucuronide – $H]^-$, 175 (B)	Urine
32	DH-RES sulfate	25.5	309	**309**/245, 229 [M – sulfate – $H]^-$ (B)	Urine
33	*cis*-RES	34.3	227	**227**/185	Urine

[*]refer to pseudo-molecular ion: $[M + HOAc - H]^-$;

[a]RES, resveratrol; RAH, resveratrol-aspirin hybrid; DA-RAH, deacetylated resveratrol-aspirin hybrid; DH-RES, dehydroresveratrol;

[b]IC, intestine contents; SC, stomach contents.

FIGURE LEGENDS

Scheme 1. Synthesis of RES-based ASA derivatives **4–10**.

Scheme 2. Synthesis of RAH resemblances **11** and **12**.

Figure 1. Structures of RES-based ASA derivatives **4–10** and RAH-like molecules **11** and **12** as well as their respective IC_{50} values against the growth of HCT-116 and HT-29 human colon cancer cells. The IC_{50} values are expressed as the mean ± SD (n = 6).

Figure 2. Dose-dependent inhibitory effect of colony formation by ASA, RES, ASA+RES, and RAH (A), and RAH, **5**, **11** and **12** (B) in HCT-116 and HT-29 cancer cells. Cells were treated with compounds and incubated in 6-well plates for 2 weeks, and the cells were then stained with crystal violet and counted for colony formation. Each column represents a mean ± SD (n=3)**.** ASA, aspirin; RES, resveratrol; RAH, resveratrol-aspirin hybrid.

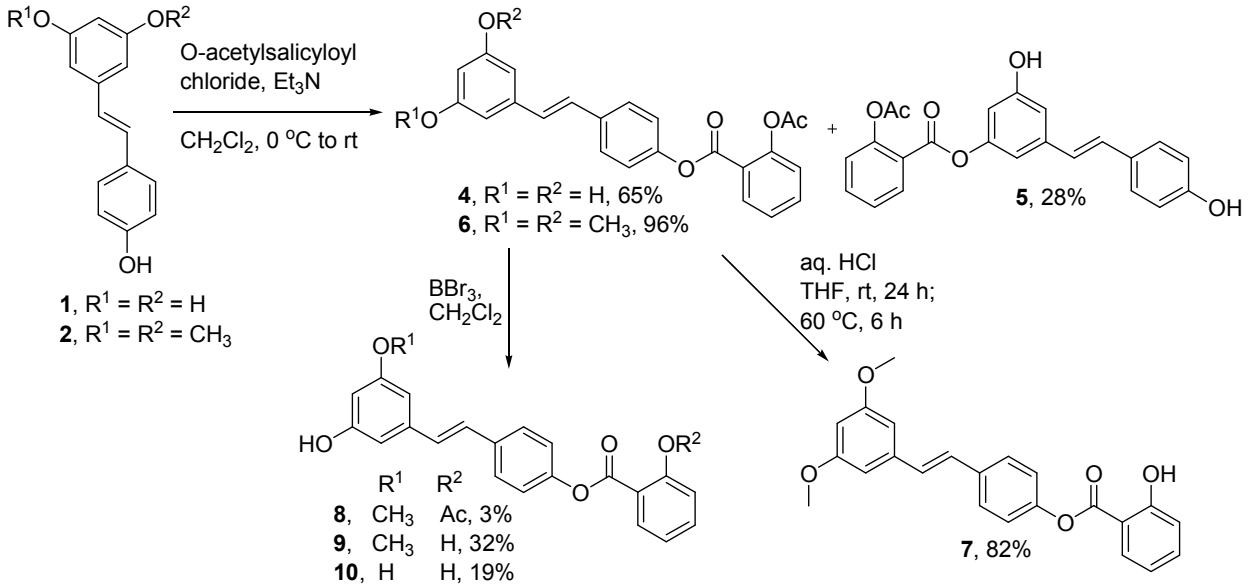
Figure 3. HPLC-ECD chromatograms of RAH in human colon cancer cells HCT-116. Cells were incubated with RAH (20 μM) at 37 ^{o}C. 100 μL of samples of supernatant were taken for each time point (0, 0.5, 1, 2, 4, 8, and 24 h). **1**, resveratrol; **4**, RAH; **10**, DA-RAH.

Figure 4. HPLC-CEAD chromatograms of standards (A), and stomach content control (B), stomach contents collected at 1 h (C) and 2 h (D), small intestine content control (E), small intestine contents collected at 1 h (F) and 2 h (G) from RAH-treated mice (200 mg/kg, intragastric gavage) ; and extracted ion chromatograms of feces (H), colon tissues collected at 1 h (I) and 2 h (J), plasma samples collected at 1 h (K) and 2 h (L), and urine before (M)/after (N) hydrolysis from RAH-treated mice (200 mg/kg, intragastric gavage) obtained by negative ESI-MS interface. **1**, RES; **4**, RAH; **10**, DA-RAH; **22**, salicyluric acid; **23**, salicylic acid; **24**, DH-RES; **25**, RES sulfate; **26**, RES diglucuronide; **27**, DH-RES diglucuronide; **28**, RES

sulfoglucuronide; **29**, DH-RES sulfoglucuronide; **30**, RES glucuronide; **31**, DH-RES glucuronide; **32**, DH-RES sulfate; **33**, *cis*-RES.

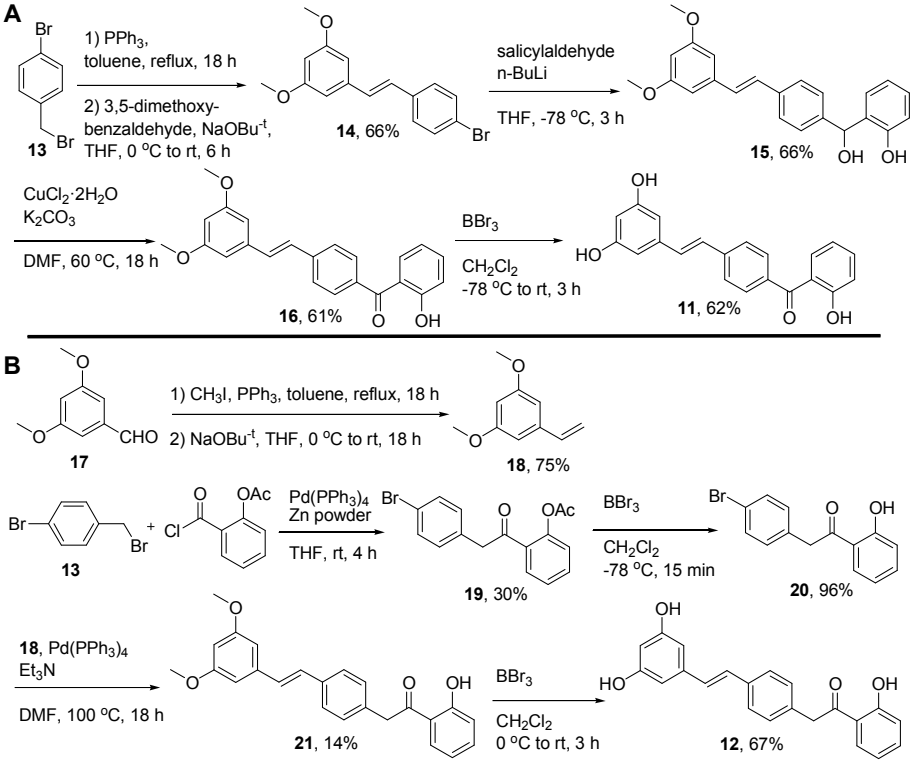
Figure 5. Effect of RAH on cell cycle arrest in colon cancer cell lines. (A) RAH causes cell cycle arrest at the G_1 phase in HCT-116 and at the G_1 and S phases in HT-29 cells. Cells were treated with RAH (0, 20, 40 and 60 μM) for 24 h. The graphs show the cell cycle distribution of propidium-iodide stained cells. The data in graph represents the percentage of cells in each stage of the cell cycle (G_0/G_1, S and G_2/M phases); (B) RAH decreases Cyclin D1 and Cyclin E protein levels in both HCT-116 and HT-29 cells. Cell extracts after treatment for 24 h with 0, 20, 40 and 60 μM of RAH, respectively, were performed to western blot analysis.

Figure 6. Effect of RAH on apoptosis in colon cancer cell lines. (A) RAH induces apoptosis in both HCT-116 and HT-29 cells. Cells treated with RAH (0, 20 and 40 μM) for 24 h were measured using PI staining; (B) RAH releases cytochrome c through regulation of Bcl2 family proteins via mitochondria; and (C) RAH activates caspase-3 expression. Cell extracts after treatment for 24 hours with respective 0, 20, 40 and 60 μM of RAH were performed to Western blot analysis.

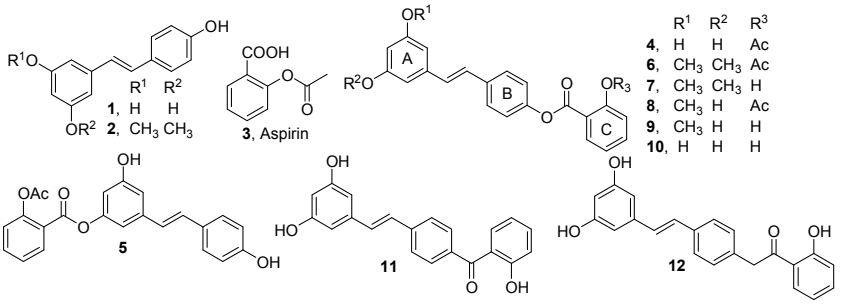


787

788 **Scheme 1.**



789
790
791 **Scheme 2.**



Compounds	IC_{50} values (μM)	
	HCT-116	HT-29
Resveratrol, **1**	> 60	> 60
Pterostilbene, **2**	> 60	> 60
Aspirin, **3**	> 60	> 60
RAH, **4**	39.39 ± 0.55	34.37 ± 0.79
5	55.84 ± 1.34	42.63 ± 1.17
6	< 60	< 60
7	55.70 ± 1.40	56.98 ± 1.54
8	< 60	< 60
9	53.59 ± 1.31	50.55 ± 3.27
DA-RAH, **10**	41.51 ± 0.23	35.52 ± 0.69
11	37.71 ± 1.21	37.48 ± 1.50
12	57.41 ± 0.81	56.04 ± 1.01

Figure 1.

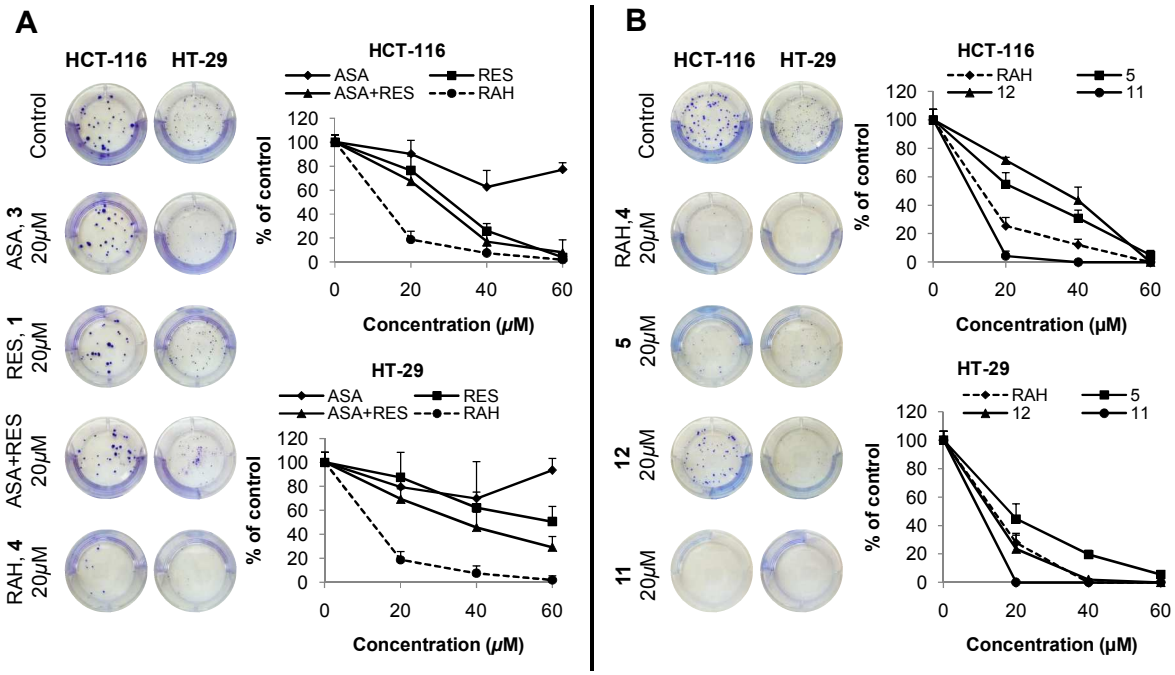

A
HCT-116
HT-29
Control
ASA, 3 20μM
RES, 1 20μM
ASA+RES 20μM
RAH, 4 20μM
HCT-116
ASA
RES
ASA+RES
RAH
% of control
Concentration (μM)
HT-29
B
HCT-116
HT-29
Control
RAH,4 20μM
5 20μM
12 20μM
11 20μM
HCT-116
RAH
5
12
11
% of control
Concentration (μM)
HT-29

Figure 2.

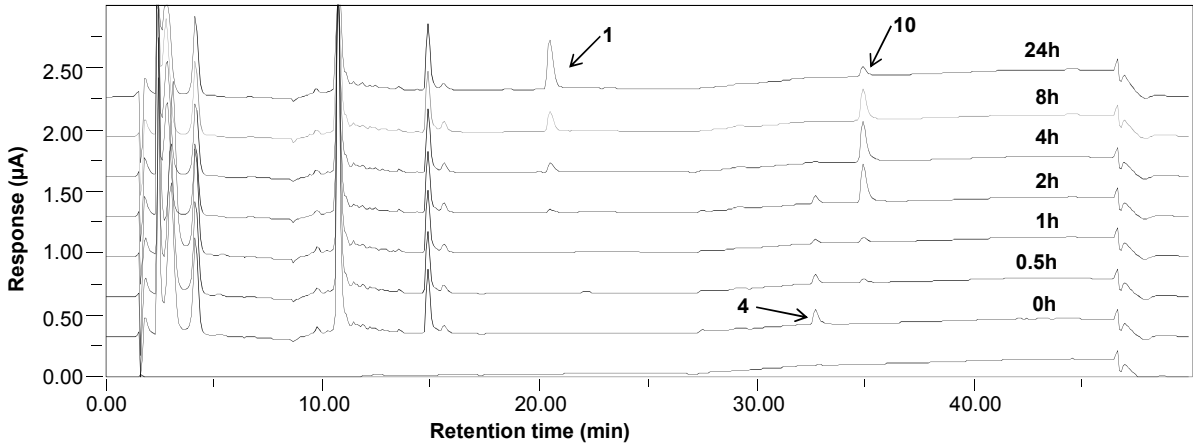

1
10
24h
8h
4h
2h
1h
0.5h
0h
4
Response (µA)
2.50
2.00
1.50
1.00
0.50
0.00
0.00
10.00
20.00
30.00
40.00
Retention time (min)

Figure 3.

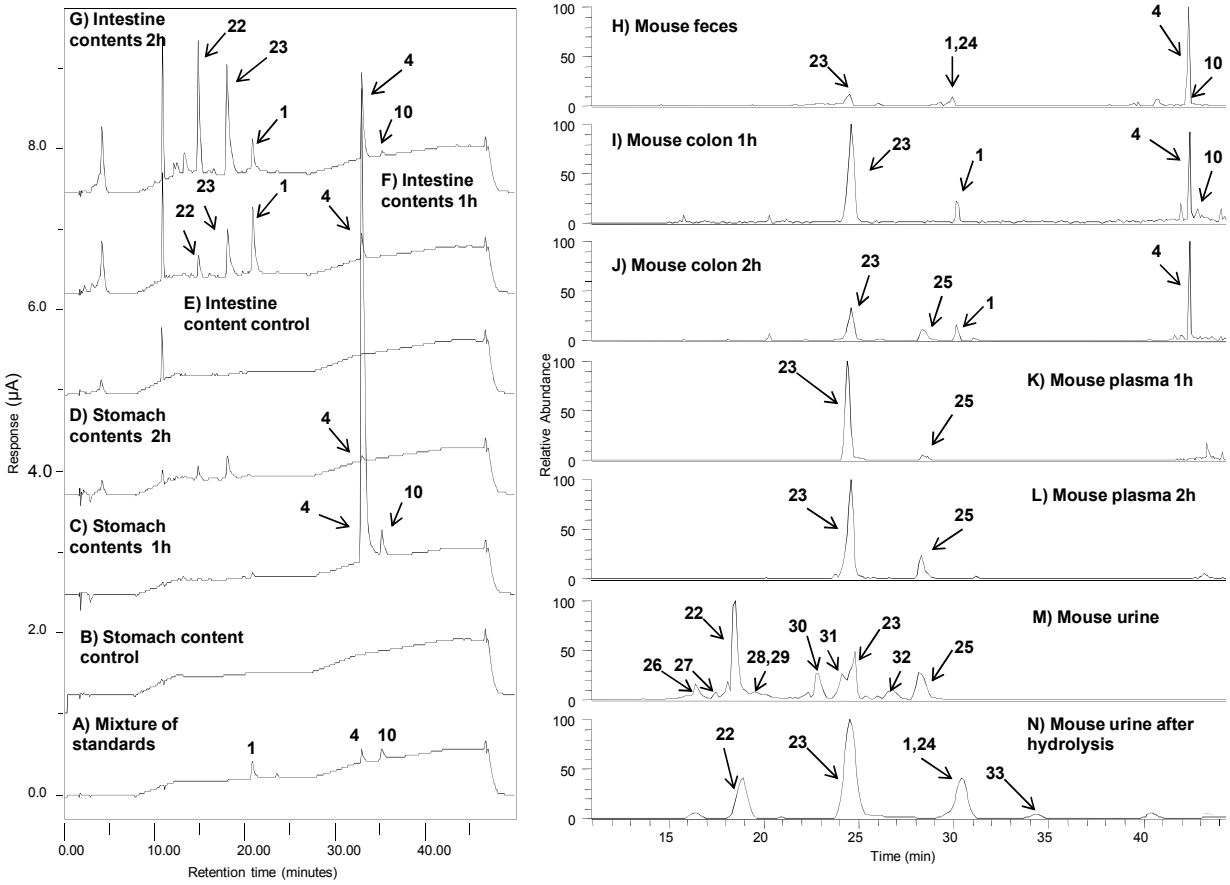

G) Intestine contents 2h
F) Intestine contents 1h
E) Intestine content control
D) Stomach contents 2h
C) Stomach contents 1h
B) Stomach content control
A) Mixture of standards
Response (μA)
Retention time (minutes)
H) Mouse feces
I) Mouse colon 1h
J) Mouse colon 2h
K) Mouse plasma 1h
L) Mouse plasma 2h
M) Mouse urine
N) Mouse urine after hydrolysis
Relative Abundance
Time (min)

Figure 4.

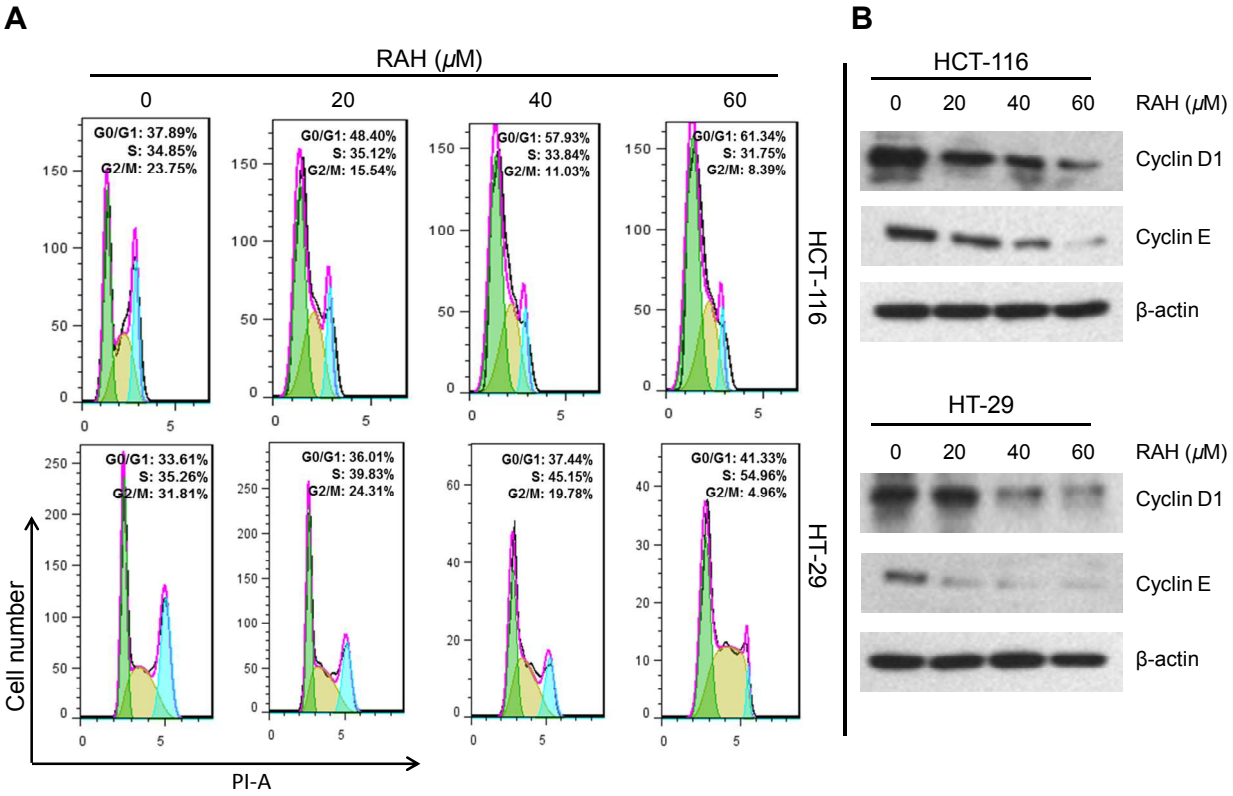

A
RAH (μM)
0
20
40
60
G0/G1: 37.89%
S: 34.85%
G2/M: 23.75%
G0/G1: 48.40%
S: 35.12%
G2/M: 15.54%
G0/G1: 57.93%
S: 33.84%
G2/M: 11.03%
G0/G1: 61.34%
S: 31.75%
G2/M: 8.39%
HCT-116
G0/G1: 33.61%
S: 35.26%
G2/M: 31.81%
G0/G1: 36.01%
S: 39.83%
G2/M: 24.31%
G0/G1: 37.44%
S: 45.15%
G2/M: 19.78%
G0/G1: 41.33%
S: 54.96%
G2/M: 4.96%
HT-29
Cell number
PI-A
B
HCT-116
0 20 40 60 RAH (μM)
Cyclin D1
Cyclin E
β-actin
HT-29
0 20 40 60 RAH (μM)
Cyclin D1
Cyclin E
β-actin

Figure 5.

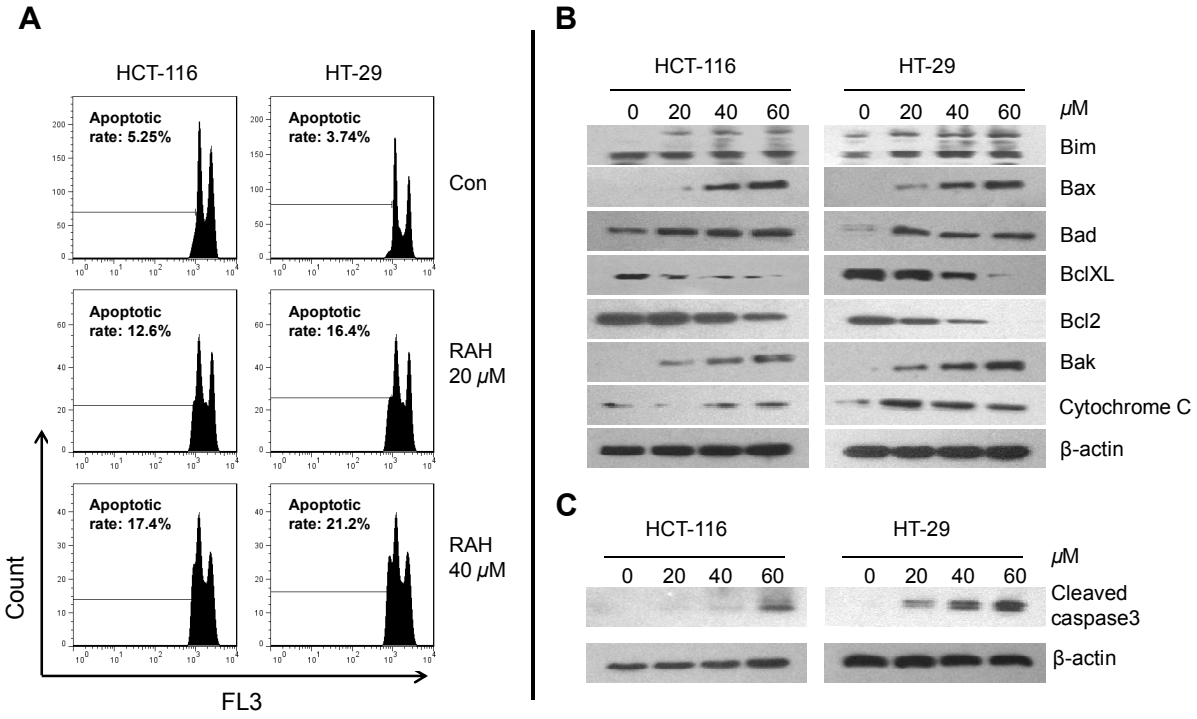
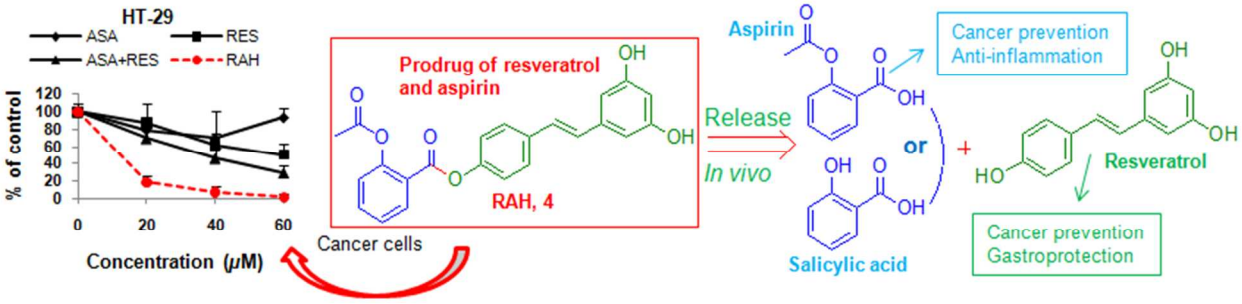

A
HCT-116
HT-29
Apoptotic rate: 5.25%
Apoptotic rate: 3.74%
Con
Apoptotic rate: 12.6%
Apoptotic rate: 16.4%
RAH 20 μM
Apoptotic rate: 17.4%
Apoptotic rate: 21.2%
RAH 40 μM
Count
FL3
B
HCT-116
HT-29
0 20 40 60
0 20 40 60
μM
Bim
Bax
Bad
BclXL
Bcl2
Bak
Cytochrome C
β-actin
C
HCT-116
HT-29
0 20 40 60
0 20 40 60
μM
Cleaved caspase3
β-actin

Figure 6.

812 **TOC GRAPHIC**



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