

Novel antioxidants are not toxic to normal tissues but effectively kill cancer cells

Anna Kovalchuk^{1,2}, Felix Aladedunye¹, Rocio Rodriguez-Juarez², Dongping Li², James Thomas², Olga Kovalchuk^{2,*}, and Roman Przybylski¹

¹Department of Chemistry; University of Lethbridge; Lethbridge, AB Canada; ²Department of Biological Sciences; University of Lethbridge; Lethbridge, AB Canada

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Free radicals are formed as a result of cellular processes and play a key role in predisposition to and development of numerous diseases and of premature aging. Recently, we reported the syntheses of a number of novel phenolic antioxidants for possible application in food industry. In the present study, analyses of the cellular processes and molecular gene expression effects of some of the novel antioxidants in normal human tissues and in cancer cells were undertaken. Results indicated that whereas the examined antioxidants showed no effects on morphology and gene expression of normal human oral and gingival epithelial tissues, they exerted a profound cell killing effect on breast cancer cells, including on chemotherapy-resistant breast cancer cells and on oral squamous carcinoma cells. Among the tested antioxidants, N-decyl-N-(3-methoxy-4-hydroxybenzyl)-3-(3,4-dihydroxyphenyl) propanamide and N-decyl-N-(3,5-dimethoxy-4-hydroxybenzyl)-3-(3,4-dihydroxyphenyl) propanamide were the most promising, with excellent potential for cancer treatment. Moreover, our gene expression databases can be used as a roadmap for future analysis of mechanisms of antioxidant action.

Introduction

Free radicals and reactive oxygen species (ROS) are formed as a result of numerous cellular processes. Their roles in health and disease have been a subject of intensive research over the past decades.¹⁻³ Free radicals and ROS cause a wide variety of effects in the cells and tissues, including DNA damage. They also mediate inflammation and immune response and alter cellular signaling processes. Furthermore, ROS cause potent induction of gene and protein expression in cells and tissues.⁴ As a result, it is currently well established that free radicals play key parts in predisposition to and development of numerous diseases, such as atherosclerosis, inflammatory joint disease, asthma, diabetes, senile dementia, Alzheimer disease, degenerative eye disease, and cancer.^{5,6} Moreover, they are also associated with premature aging.^{5,7,8}

Free radicals and their roles in food have been extensively studied as well, especially in regards to oxidative and thermooxidative deterioration of polyunsaturated fatty acids.⁹ Although synthetic antioxidants, such as butylated hydroxy toluene (BHT), butylated hydroxy anisole (BHA), and tertiary-butylatedhydroquinone (TBHQ), have shown good efficiency, their use has been hampered due to their possible detrimental effect on human health. Generally, in addition to demonstrating high efficiency, an ideal antioxidant for food application must exhibit no or significantly low toxicity.

Phenolic compounds, such as phenolic acids, tocopherol, and flavonoids, are the most widely used natural antioxidants,

principally because of their high radical scavenging activity and low toxicity. Additionally, they exhibit a spectrum of biological activity, including antiviral, anti-bacterial, anti-hypertension, anti-thrombosis, anti-fibrosis, and even anti-cancer properties.¹⁰ Therefore, the development of potent and safe phenolic antioxidants is always a worthwhile task. Recently, we reported the synthesis of 21 novel phenolic antioxidants using precursors naturally present in oil seeds.^{7,10}

Establishing safety and effectiveness of novel compounds is a key aspect in antioxidant research. Even though there has been a long history of the use of antioxidants for food preservation and other purposes, there remains an important concern about their possible effects on human health and their overall significance for human health.¹¹ Indeed, for some dietary phytochemicals, their antioxidant properties are less important for human health than the other effects that they may cause, including changes in gene expression and in cell signaling.¹¹

In sum, there currently is a significant interest in the field of antioxidant research. There still exists an important concern about the possible effects of antioxidants on human health and their overall significance for human health. Therefore, establishing safety and effectiveness of novel compounds is a key aspect of antioxidant research.

Here, we aimed to analyze the cellular and molecular effects of selected novel antioxidants in normal human tissues and in cancer cells. We, for the first time, show that while being non-toxic for normal tissues (human buccal and gingival tissues), the

*Correspondence to: Olga Kovalchuk; Email: olga.kovalchuk@uleth.ca
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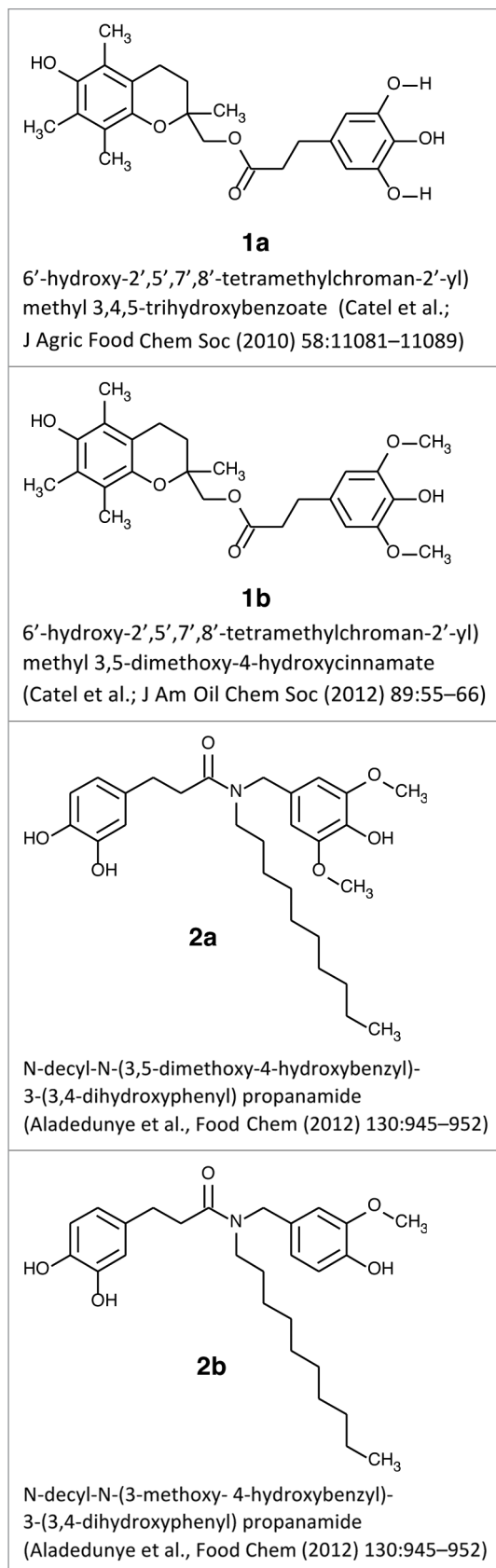


Figure 1. Studied antioxidant compounds.

examined antioxidants exerted a profound cell killing effects on oral and breast cancer cells, including chemotherapy-resistant breast cancer cells.

Results and Discussion

The key goal of this study was to determine if new lipid antioxidants exert any detrimental effects on human cells and tissues. In the present studies, we focused on four of the recently reported novel antioxidants: trolox trihydroxybenzoate (**1a**)¹²; trolox 3,5-dimethoxy-4-hydroxycinnamate (**1b**)¹³; and derivatives of dihydrocaffeic acid amide, (**2a**) and (**2b**)¹⁰ (Fig. 1). Based on the literature suggestions,¹¹ we specifically focused our study on gene expression and signaling effects of the novel antioxidants.

Effects of antioxidants on normal tissues. First, we evaluated the toxicity of the phenolic antioxidants on normal human oral and gingival epithelial tissues, since they are the first to be exposed to food and its constituents, including antioxidants. We conducted Illumina microarray-based gene expression profiling to determine gene expression patterns upon exposure of EpiOral and EpiGingival tissues to selected antioxidants. The summary of the gene expression results is presented in Table 1.

Effects in EpiOral model. Our analysis revealed that treatment with **1b** led to altered expression of 12 genes when a 1.5-fold cut-off was applied. Yet, when a more conservative 2-fold cut-off was used, only 1 gene was deregulated (Table 1; Table S1). Treatment with **2a** led to deregulated expression of 15 genes at the 1.5-fold cut-off level and only 3 genes at a 2-fold cut-off (Table 1; Table S2). Interestingly, treatment with **2b** affected 5 genes when the 1.5-fold cut-off was applied, while none of these genes changed their expression more than 2 fold (Table 1; Table S3). Among all the studied compounds, changes induced by exposure to **1a** were the most pronounced; overall, when 1.5 was used as a cut-off level, exposure to **1a** influenced expression of 52 genes. Among those, expression of 10 genes was changed more than 2 fold (Table 1; Table S4). A direct relationship between their effects on gene expression and the number of oxygenated substituents, especially the hydroxy functional group, was evident; trolox derivative **1a** has 4 OHs, whereas the much milder **1b** has only 2 OHs. Although the dihydrocaffeic acid amides both have 3 OHs, the more active **2a** possesses 2 additional methoxy substituents in comparison to the 1 methoxy group in **2b**.

Even though **1a** affected the expression of many genes, some of these gene expression changes may be viewed as protective. Among those, **1a** suppressed the levels of the MYC oncogene, CDK5, and other genes involved in oncogenesis (Table S4). It also downregulated the MMP10 gene, which codes for a protein involved in cell–cell communication and metastasis.¹⁴ Interestingly, a recent study has shown that eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) inhibit gastric cancer cell migration by suppressing the expression of matrix metalloproteinase 10.¹⁴

Among the numerous genes altered by exposure of EpiOral tissues to antioxidants **1a**, **1b**, **2a**, and **2b**, only one gene was common in all four groups, ADRB2. This gene encodes a β -2-adrenergic receptor, which is a member of the G protein-coupled

receptor super-family that is reported to be important in salivary gland function.¹⁵ Although the roles of ADRB2 in the function of oral epithelium are unclear,¹⁶ a recent study indicated that it might be involved in wound healing responses of oral epithelial cells.¹⁶

Four genes were common between the 1a and 2a groups; among those is the EGR1 gene. The protein encoded by this gene belongs to the EGR family of C₂H₂-type zinc-finger proteins. It is a nuclear protein and functions as a transcriptional regulator. It activates numerous genes, the products of which are required for differentiation and mitogenesis, such as PTEN, p53, and fibronectin.¹⁷ EGR1 is considered an important tumor suppressor.¹⁷ Therefore, the fact that EGR1 is upregulated by antioxidants in normal oral tissues may be viewed as a positive protective effect.

The DDIT4 gene encodes for protein regulation in development and DNA damage responses. Specifically, it inhibits cell growth by regulating the TOR signaling pathway upstream of the TSC1-TSC2 complex.¹⁸ Therefore, this gene has a protective, growth inhibitory effect. Another commonly changed gene is ADM. It encodes a vasodilator peptide, and its role in antioxidant response needs to be further established. The function of the ankyrin repeat domain 37 gene remains unknown.

Effects in EpiGingival tissue. Our analysis revealed that treatment of EpiGingival tissues with 1b led to altered expression of 52 genes when a 1.5-fold cut-off was applied. Yet, when a more conservative 2-fold cut-off was used, only 3 genes were deregulated (Table 1; Table S5). Treatment with 2a led to deregulated expression of 131 genes at the 1.5-fold cut-off level, and only 5 genes at a 2-fold cut-off level (Table 1; Table S6). Interestingly, treatment with 2b affected 8 genes when the 1.5-fold cut-off was applied, while only one of these genes changed its expression more than 2-fold (Table 1; Table S7). Treatment with 1a led to altered expression of 15 genes when the 1.5-fold cut-off level was used. Upon employment of a more conservative 2-fold cut-off, only 3 genes were deregulated (Table 1; Table S8).

Interestingly, in EpiGingival tissues among all four studied compounds, changes induced by exposure to 2a were the most pronounced. However, in both tissues, compound 2b induced the least amount of changes (Table 1).

Having observed changes in gene expression induced by antioxidants in normal oral tissues, we further proceeded to analyze the levels of several proteins involved in cell proliferation and damage responses (Fig. 2). We focused on the proliferating cells' nuclear antigen (PCNA), phosphorylated histone H2AX (γ H2AX), meiotic recombination 11 homolog A (MRE11), and p21/WAF1, a cyclin-dependent kinase inhibitor (p21).¹⁹⁻²¹

PCNA is a well-established marker of S-phase cells. Phosphorylated histone H2AX is a marker of DNA damage. Histone H2AX is rapidly phosphorylated at Ser¹³⁹ upon induction of DNA strand breaks, and it can be effectively detected using specific antibodies.^{22,23} Analysis of histone H2AX phosphorylation is widely used to assess the extent of damage to cellular DNA.²⁴ MRE 11, on the other hand, is a key DNA repair protein.²⁵ P21 is one of the key tumor suppressors and plays

Table 1. Summary of the gene expression in EpiOral and EpiGingival tissues upon treatment with novel antioxidant compounds 1a, 1b, 2a, and 2b

Antioxidant compounds	Gene expression fold change as compared with control			
	1.5-fold cut-off		2-fold cut-off	
	EpiOral	EpiGingival	EpiOral	EpiGingival
1a	51	15	10	3
1b	12	52	1	3
2a	15	131	1	5
2b	5	8	0	1

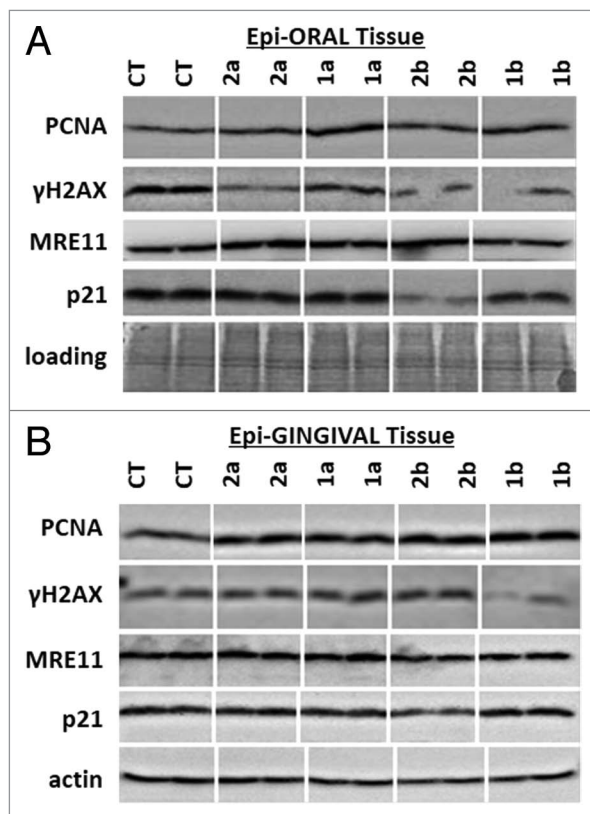


Figure 2. Expression of PCNA, γ H2AX, MRE11 and p21 in EpiOral (A) and EpiGingival (B) tissues treated with 1a, 1b, 2a, and 2b antioxidants. CT, control tissues; 1a, 1b, 2a, 2b, tissues treated with antioxidants. Antioxidants: 1a, 6'-hydroxy-2',5',7',8'-tetramethylchroman-2'-yl) methyl 3,4,5-trihydroxybenzoate; 1b, 6'-hydroxy-2',5',7',8'-tetramethylchroman-2'-yl) methyl 3,5-dimethoxy-4-hydroxycinnamate; 2a, N-decyl-N-(3,5-dimethoxy-4-hydroxybenzyl)-3-(3,4-dihydroxyphenyl) propanamide; 2b, N-decyl-N-(3-methoxy-4-hydroxybenzyl)-3-(3,4-dihydroxyphenyl) propanamide.

numerous essential roles in DNA damage response, cell cycle arrest, apoptosis, and transcription.²⁶

In EpiOral tissue, we noted that exposure to antioxidants did not lead to any significant changes in the levels of PCNA and MRE11 (Fig. 2A). The levels of γ H2AX were reduced by 2b, 1b, and 1a compounds (Fig. 2A). This is an interesting observation, as γ H2AX is usually induced upon DNA damage.²⁷ Therefore, a

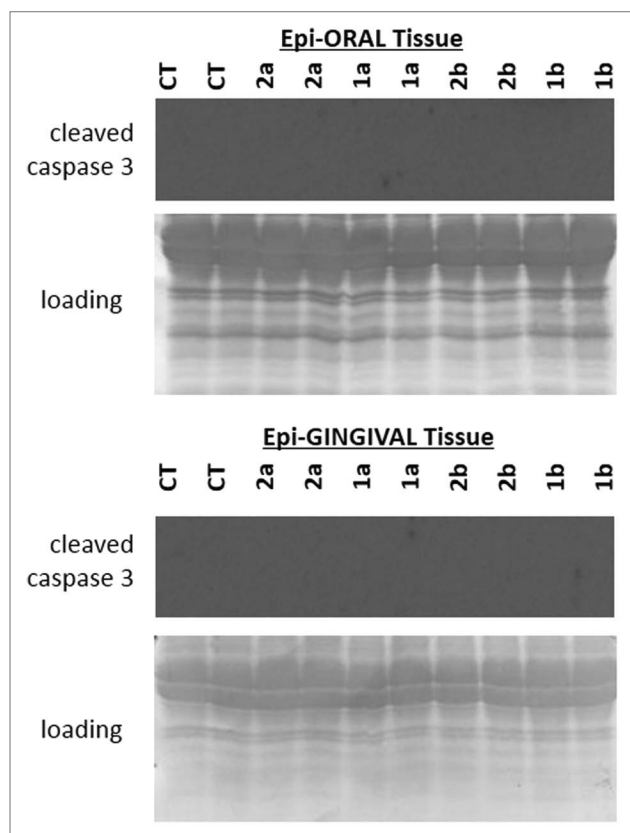


Figure 3. Levels of cleaved caspase 3 in EpiOral and EpiGingival tissues treated with **1a**, **1b**, **2a**, and **2b** antioxidants. CT, control tissues; **1a**, **1b**, **2a**, **2b**, tissues treated with antioxidants. Antioxidants: **1a**, 6'-hydroxy-2',5',7',8'-tetramethylchroman-2'-yl) methyl 3,4,5-trihydroxybenzoate; **1b**, 6'-hydroxy-2',5',7',8'-tetramethylchroman-2'-yl) methyl 3,5-dimethoxy-4-hydroxycinnamate; **2a**, N-decyl-N-(3,5-dimethoxy-4-hydroxybenzyl)-3-(3,4-dihydroxyphenyl) propanamide; **2b**, N-decyl-N-(3-methoxy-4-hydroxybenzyl)-3-(3,4-dihydroxyphenyl) propanamide.

decrease in γ H2AX levels may be viewed as a positive, protective effect.

Interestingly, we noted that compound **1a** strongly suppressed p21 in EpiOral tissues. Notwithstanding, gene expression data did not show any changes in the levels of p21 gene expression. Thus, its expression may be regulated posttranscriptionally. Indeed, small RNAs may effectively suppress the protein levels.²⁸ In the future, it would be interesting to analyze the effects of the selected antioxidants on the small RNA levels and to correlate those with the levels of gene expression and protein levels.

Similar to observations in EpiOral tissues, we did not see any changes in the levels of MRE11 and p21 in EpiGingival tissues upon exposure to antioxidants (Fig. 2B). Levels of γ H2AX were unaffected by **1a**, **2a**, and **2b** compounds; however, exposure to antioxidant **1b** led to a decrease in the levels of γ H2AX, suggesting a protective effect similar to the one observed in EpiOral tissues.

Additionally, we have analyzed the level of cleaved caspase 3 as an established marker for apoptosis.²⁹ None of the studied antioxidants led to caspase 3 cleavage. The apparent lack of caspase 3

cleavage indicates that novel antioxidants do not cause cell death in normal oral and gingival tissues (Fig. 3).

Overall, based on the gene expression and protein expression levels, we suggest that the studied novel antioxidants are not toxic to the oral and gingival epithelial tissues, and some of the noticed changes may in fact be protective in nature. In the future, studies will be needed to conduct a further in-depth analysis of molecular, cellular, and histological effects of these antioxidants on a wide array of normal tissues as a function of time and concentration.

Effects of antioxidants on cancer cells. Having seen no toxic effects of antioxidants on normal oral and gingival cells, we proceeded to analyze their effects on tumor cells. In the present study, we used three oral squamous cell carcinoma lines—CAL-27, UMSSC1, and UMSSC47. CAL-27 is a tongue squamous cell carcinoma; UMSSC1 and UMSSC47 are oral cavity squamous carcinoma cell lines.³⁰

We noted that in cell line CAL27, the levels of γ H2AX were upregulated by **2b**, **2b**, and **1a** compounds (Fig. 4). Among them, **2b** caused the highest upregulation of γ H2AX. Similarly, in the oral cavity cancer cell line UMSSC47, exposure to **2a**, **2b**, and **1a** compounds resulted in a strong upregulation of γ H2AX. Contrarily, in the UMSSC1 line, γ H2AX phosphorylation was increased only upon exposure to compound **2a**. The increased levels of γ H2AX may be indicative of DNA damage; therefore future studies are needed to dissect the precise nature of DNA damage induced by novel antioxidants in cancer cells.

Our analysis also showed that exposure to antioxidants did not lead to any significant changes in the levels of p21 and MRE11 in all studied OSCC lines. The levels of PCNA were affected only in UMSSC1 upon exposure to **2b** compound that caused a slight decrease in PCNA levels.

But most interestingly, we found that novel antioxidants **2a** and **2b** caused a profound increase in the levels of cleaved caspase 3 in CAL 27 and UMSSC47 cells. In UMSSC1 cells, caspase 3 cleavage was caused only by **2b** compound. Caspase-3 is a critical executioner of apoptosis, and the induction of caspase 3 cleavage is a well-established indicator of apoptosis²⁹ (Fig. 4).

To gain further insight into the apoptosis-inducing potential of **2a** and **2b**, we analyzed the level of apoptosis in OSCC cell lines by the Annexin V assay. Our analysis revealed that both compounds caused a profound induction of apoptosis in the OSCC cell lines (Fig. 5). The profound apoptosis-inducing effects of novel antioxidants indicate their excellent potential as novel anti-cancer regimens.

To analyze the anticancer effects of the novel antioxidants further, we used human breast adenocarcinoma MCF-7 cells, an ER-positive, hormone-sensitive cell line that represents early stage human breast cancer. In parallel, we used MCF-7 variants resistant to common chemotherapy drugs, doxorubicin (MCF-7/DOX) and *cis*-dichlorodiammine platinum (II) or cisplatin (MCF-7/CIS).³¹ The drug-resistant variants of the MCF-7 cell lines were previously established by stepwise selection after prolonged (>6 mo) treatment of the original MCF-7 cells with escalating concentrations of DOX or CIS at a range of 0.5 to 15 μ g/mL in the medium. After 6 mo of culturing in the presence of

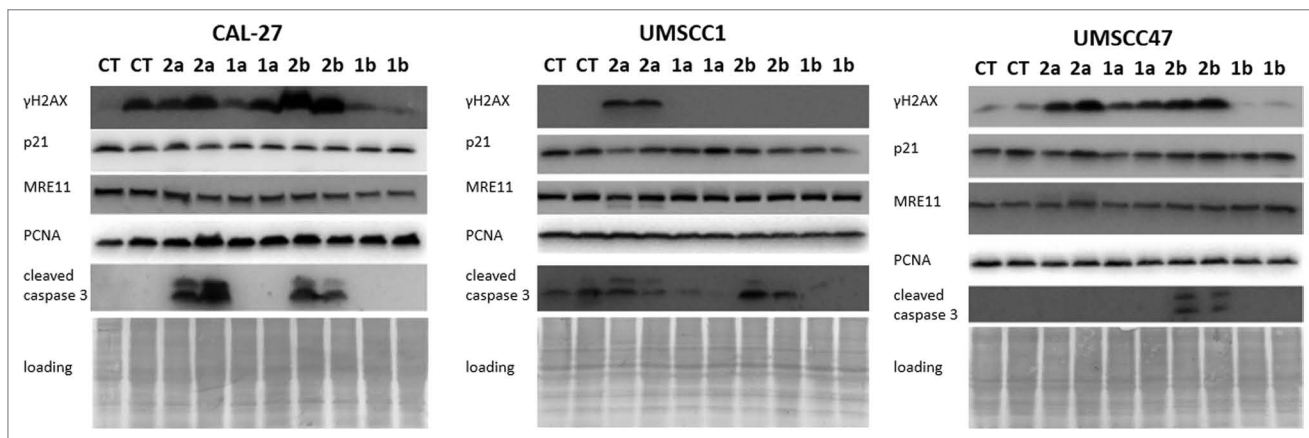


Figure 4. Expression of γ H2AX, PCNA, MRE11, p21, and cleaved caspase 3 in CAL27, UMSSC1 and UMSSC47 oral squamous cell carcinoma lines treated with **1a**, **1b**, **2a**, and **2b** antioxidants. CT, control tissues; **1a**, **1b**, **2a**, **2b**, tissues treated with antioxidants. Antioxidants: **1a**, 6'-hydroxy-2',5',7',8'-tetramethylchroman-2'-yl) methyl 3,4,5-trihydroxybenzoate; **1b**, 6'-hydroxy-2',5',7',8'-tetramethylchroman-2'-yl) methyl 3,5-dimethoxy-4-hydroxycinnamate; **2a**, N-decyl-N-(3,5-dimethoxy-4-hydroxybenzyl)-3-(3,4-dihydroxyphenyl) propanamide; **2b**, N-decyl-N-(3-methoxy-4-hydroxybenzyl)-3-(3,4-dihydroxyphenyl) propanamide.

drugs, the IC_{50} (inhibitory concentration to produce 50% cell death) values were 19.2 and 3.6 mg/L for the MCF-7/DOX and MCF-7/CIS cells, respectively.

At 70% confluency, the cells were treated with the studied antioxidant compounds at a concentration of 350 μ g/mL for 2 h. Interestingly, we noted that all cells were dead after 30 min of such exposure. The experiment was reproduced a second time, and the same phenomenon of total cell death was observed.

Next, we reduced the concentration of antioxidants to 117 μ g/mL. After 2 h of treatment, the cells were analyzed for survival and for gene expression. As with normal tissues, we used Illumina HumanHT-12 v4 Expression BeadChip arrays. At the same concentrations that did not cause any effects on normal cells, antioxidants led to significant alterations in gene expression in cancer lines.

Exposure of MCF-7 to **1b** led to changes in expression of 28 genes. Application of **2b** to MCF-7 cells altered the expression of 845 genes. Interestingly, **1a** and **2a** were so toxic to the cells that, even though we were able to extract the RNA from the cells, the RNA did not yield any good cDNA libraries. This is indicative of apoptotic-related processes and further confirms a very profound and rapid death of MCF-7, DOX and CIS cells induced by antioxidants.

Compounds **1a** and **1b** affected gene expression in DOX and CIS cells as well. Indeed, exposure of DOX cells to **1b** resulted in altered expression of 50 genes, and exposure to **1a** led to altered expression of 7 genes. Three genes were common in both groups (Table S9). In CIS cells, **1a** induced expression changes of 56 genes, and **1b** induced changes of 24 genes. Three genes were common between **1a**- and **1b**-exposed CIS cells (Table S10). Interestingly, compounds **2a** and **2b** were extremely toxic to DOX and CIS cells.

Overall, we noted that **2b** affected expression of MCF-7 cells, but killed DOX and CIS cells. Therefore, among all the studied compounds, **2b** appeared to be the most interesting and

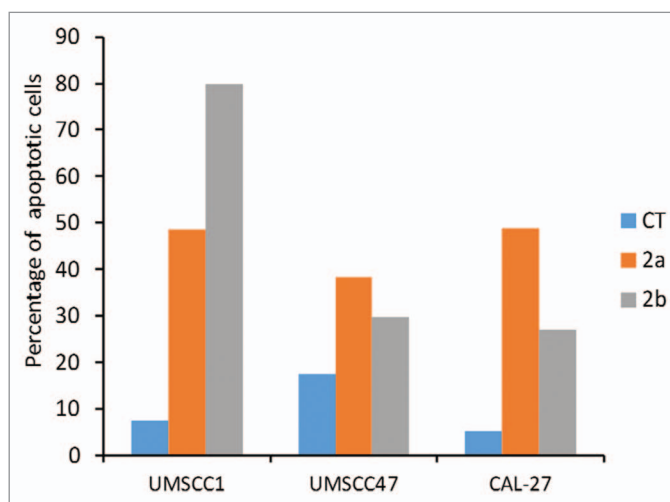


Figure 5. Induction of apoptosis by **1a**, **1b**, **2a**, and **2b** antioxidants in CAL27, UMSSC1 and UMSSC47 oral squamous cell carcinoma lines as analyzed by the Annexin V assay. CT, control tissues; **1a**, **1b**, **2a**, **2b**, tissues treated with antioxidants. Antioxidants: **1a**, 6'-hydroxy-2',5',7',8'-tetramethylchroman-2'-yl) methyl 3,4,5-trihydroxybenzoate; **1b**, 6'-hydroxy-2',5',7',8'-tetramethylchroman-2'-yl) methyl 3,5-dimethoxy-4-hydroxycinnamate; **2a**, N-decyl-N-(3,5-dimethoxy-4-hydroxybenzyl)-3-(3,4-dihydroxyphenyl) propanamide; **2b**, N-decyl-N-(3-methoxy-4-hydroxybenzyl)-3-(3,4-dihydroxyphenyl) propanamide.

promising one; it did not affect normal tissues, but it causes profound deregulation of gene expression in breast adenocarcinoma tissues and effectively eradicated drug resistant cells.

Treatment with **2b** led to altered expression of 845 genes, thus affecting numerous cellular pathways (Table S11). It caused upregulation of the MAPK signaling pathway (Fig. 6). Additionally, it downregulated key metabolic pathways, such as oxidative phosphorylation, ribosome, aminoacyl-tRNA biosynthesis, citrate cycle (TCA cycle), proteasome, spliceosome, fatty

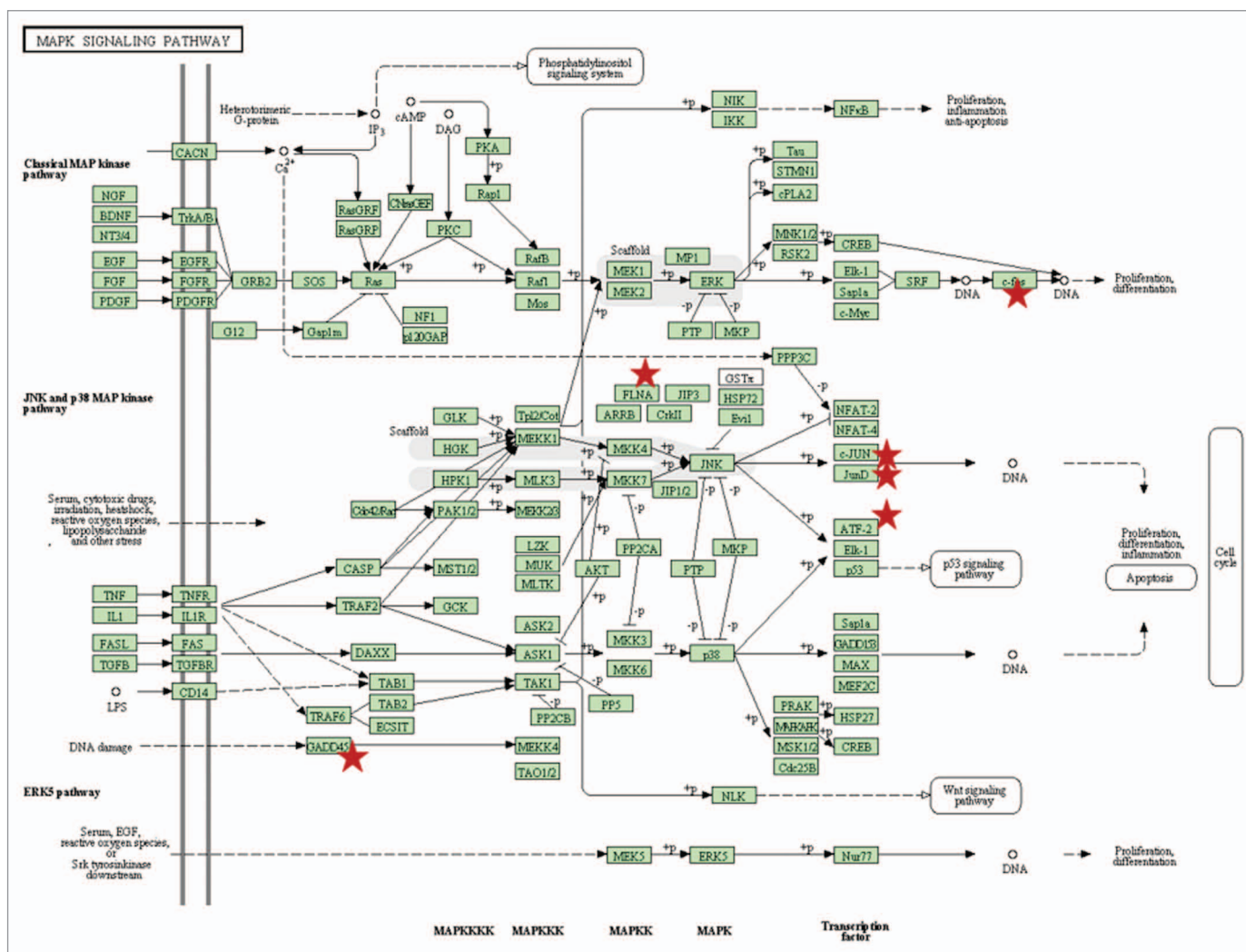


Figure 6. Schematic representation of the MAPK pathways that is upregulated in MCF-7 cells upon treatment with compound **2b** (N-decyl-N-[3-methoxy-4-hydroxybenzyl]-3-[3,4-dihydroxyphenyl] propanamide). DAVID Bioinformatic resource (<http://david.abcc.ncifcrf.gov/>), the KEGG Pathways.⁴² The KEGG (Kyoto Encyclopedia of Genes and Genomes) MAPK pathway map can be found online at <http://www.genome.jp/kegg/>. Stars denote genes which exhibited altered expression upon antioxidant treatment.

acid elongation in mitochondria, glutathione metabolism, and the pyruvate metabolism pathways (Table 2). These pathways are critically important for general cellular wellbeing, and their downregulation may lead to cell death. Indeed, altered functioning of these key networks may underlie the mechanisms of antioxidant-induced killing of MCF-7 cells.

Future Perspectives

Based on the data presented herein, compounds **2a** and **2b** seem to be the most promising ones. They spare the normal cells, but effectively kill both oral cavity and breast cancer cells. Moreover, compound **2b** is especially active in killing multi-drug resistant breast cancer cells. The latter finding is of crucial importance, as multi-drug resistance is a major clinical problem.³¹ It usually leads to tumor recurrence and a poor clinical outcome. In the future, it would be important to conduct a detailed analysis of the

potential of compound **2b** for killing multi-drug resistant cells and tumors of other tissue types, including colon, lung, melanoma, prostate cancer.

Of special interest and importance would be applicability of compounds **2a** and **2b** to treat oral squamous cell carcinoma (OSCC). Since compounds **2a** and **2b** are non-toxic against normal oral and gingival tissues and effectively induced DNA damage and apoptosis in OSCC cell lines, in the future, these compounds should be further investigated for their potential in oral cancer treatment. OSCC accounts for approximately 300,000 new cases worldwide, including 30,000 in North America, annually. This debilitating tumor is very difficult to treat and has a dismal prognosis.^{32,33} Despite advances in diagnostic, surgical, and chemo-radiation techniques, only 40–50% of OSCC patients survive beyond 5 years—in fact, survival rates in OSCC have remained unchanged for the last five decades. Even patients surviving OSCC often endure chronic and debilitating

Table 2. List of pathways differentially regulated in MCF-7 cells treated with 2b compound

Upregulated		
Term	P value	Altered genes
hsa05322:Systemic lupus erythematosus	$P < 0.0001$	HIST2H2AA3, HIST1H2BC, HIST1H2BD, HIST1H2BE, HIST1H2BF, HIST1H2BG, HIST1H2AE, HIST1H2BH, H2AFJ, HIST2H3C, HIST2H3D, HIST2H2AB, HIST2H2BE, HIST2H2AC, HIST1H2BJ, HIST1H4E, HIST1H2AH, HIST1H3D, HIST1H3F, HIST1H3G, HIST1H3H, HIST1H4H
hsa00900:Terpenoid backbone biosynthesis	$P < 0.0001$	MVD, HMGCS1, FDPS, IDI1
hsa04010:MAPK signaling pathway	$P < 0.0001$	FOS, ATF4, JUN, JUND, HSPA6, HSPA1A, HSPA1B, GADD45B, FLNA, DDIT3, ATF2
Downregulated		
Term	P value	Altered genes
hsa05012:Parkinson disease	$P < 0.0001$	ATP5D, NDUFB5, UQCRC1, COX7A2, NDUFA8, LOC729317, NDUFA9, NDUFB9, COX8A, COX7A2L, COX5A, COX5B, PARK7, VDAC1, LOC402175, SDHD, COX6A1, UBB
hsa00190:Oxidative phosphorylation	$P < 0.0001$	ATP5D, NDUFB5, UQCRC1, COX7A2, NDUFA8, NDUFA9, COX10, NDUFB9, COX8A, COX7A2L, COX5A, COX5B, ATP6V1A, ATP6V0E2, LOC402175, SDHD, COX6A1
hsa05010:Alzheimer disease	$P < 0.0001$	ATP5D, NDUFB5, COX7A2, UQCRC1, NDUFA8, APH1A, NDUFA9, NDUFB9, COX8A, COX7A2L, COX5A, COX5B, ATP2A2, LOC402175, SDHD, PPP3CB, COX6A1, CHP, CALM1
hsa05016:Huntington disease	$6.21E-04$	ATP5D, NDUFB5, UQCRC1, COX7A2, NDUFA8, LOC729317, NDUFA9, NDUFB9, COX8A, COX7A2L, COX5A, COX5B, DCTN2, VDAC1, LOC402175, SDHD, CREB3L2, COX6A1, AP2M1
hsa03010:Ribosome	0.001104	MRPL13, RPS3A, RPL34, RPL8, RPL15, RPL27, RPS13, FAU, RPL24, RPL7A, RPS11, RPS3
hsa00970:Aminoacyl-tRNA biosynthesis	0.001563	WARS, YARS, DARS, EPRS, IARS2, DARS2, KARS, EARS2
hsa00020:Citrate cycle (TCA cycle)	0.009441	SUCLG2, SUCLG1, SDHD, IDH1, PDHB, MDH1
hsa03050:Proteasome	0.0143	PSMB5, PSMB7, PSMD14, PSMB6, PSMA6, PSMB2, PSMD2
hsa04142:Lysosome	0.027671	LAMP1, LAMP2, AP4E1, AP1G1, PSAP, IGF2R, HEXB, PPT1, SCARB2, GBA, AP3B1
hsa03040:Spliceosome	0.042939	LOC100129585, EFTUD2, ZMAT2, SF3B5, RBMX, CTNBNL1, PRPF19, SFRS7, PLRG1, SFRS9, SNRPC, LOC100131735, PUF60
hsa00062:Fatty acid elongation in mitochondria	0.043188	ACAA2, PPT1, HADHB
hsa04260:Cardiac muscle contraction	0.048592	COX7A2, UQCRC1, ATP2A2, COX8A, COX6A1, COX7A2L, COX5A, COX5B
hsa00860:Porphyrin and chlorophyll metabolism	0.050819	BLVRA, COX10, FTHL3, EPRS, EARS2
hsa00480:Glutathione metabolism	0.061389	MGST3, GPX4, RRM1, PGD, LOC642590, IDH1, LOC646347, SMS
hsa05120:Epithelial cell signaling in <i>Helicobacter pylori</i> infection	0.069935	ATP6V1A, TJP1, ATP6V0E2, MAPK13, RAC1, NFKB1, PTPN11
hsa05014:Amyotrophic lateral sclerosis (ALS)	0.075133	DERL1, MAPK13, RAC1, PPP3CB, BCL2L1, CHP
hsa04722:Neurotrophin signaling pathway	0.083546	MAP2K1, MAPK13, RAC1, YWHAB, YWHAQ, RHOA, NFKB1, CRK, CALM1, PTPN11
hsa00620:Pyruvate metabolism	0.090327	ME1, LDHA, GLO1, PDHB, MDH1
hsa04114:Oocyte meiosis	0.098472	PPP1CA, MAP2K1, YWHAB, YWHAQ, PPP3CB, PPP2R5E, CHP, PPP1CC, CALM1

consequences of treatment. These novel antioxidants may hold potential for cancer treatment.

Furthermore, it would be interesting to check the protective effects of novel antioxidants. Sometimes, an application of a given substance may lead to a decrease in the rates of carcinogen-induced cancer. Would the same apply for pre-treatment of cells with novel antioxidant compounds? This question remains to be answered. Additionally, animal studies are clearly needed to analyze whole organism and tissue-specific effects of novel antioxidants.

Materials and Methods

Antioxidants. The syntheses of the novel compounds have previously been reported: **1a**,¹² **1b**,¹³ **2a**, and **2b**¹⁰ (Fig. 1).

Tissues and treatment. We used EpiOral™ and EpiGingival™ tissues (Mattek Inc.). EpiOral™ tissues are 3-dimensional, highly differentiated, metabolically and mitotically active tissues. They are produced from normal human cells and represent buccal (inner cheek) cell phenotypes. EpiOral tissues are multilayered with organized basal layer and multiple non-cornified layers, harbor in vivo-like lipid profiles, produce human β defensins, and overall are known to be ideal for irritation, toxicity, and oral pathology studies.

The EpiGingival tissue is also multilayered except that the apical layers are cornified, similar to in vivo gingival tissue. Both of these tissue models have been used to study effects of DNA damaging agents³⁴ as well as toxic effects of ethanol and the mouth rinse Listerine.³⁵

EpiOral and EpiGingival tissues were cultured as recommended by the manufacturer (Mattek Inc.). Tissues were treated with 117 $\mu\text{g}/\text{mL}$ of aforementioned antioxidants in the culture media for 2 h.

Cell lines and treatment. To analyze potential anti-cancer effects of novel antioxidants, we used three oral squamous cell carcinoma lines—CAL-27, UMSCC1, and UMSCC47. CAL-27 is a tongue squamous cell carcinoma; UMSCC1 and UMSCC47 are oral cavity squamous carcinoma cell lines.^{30,36,37} These lines were a kind gift of Professor Joseph Dort. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/High Glucose (Hyclone) with 100 nmol/L nonessential amino acids (Gibcon) and 2 mmol/L L-glutamine (Hyclone). All culture media were supplemented with 10% fetal bovine serum (Gibco), as recommended.

Additionally, we used human breast adenocarcinoma MCF-7 cells, an ER-positive, hormone-sensitive cell line that represents early stage human breast cancer. In parallel to MCF-7, we used MCF-7 variants resistant to common chemotherapy drugs—doxorubicin (MCF-7/DOX) and *cis*-dichlorodiammine platinum(II) or cisplatin (MCF-7/CIS).³¹ The cells were cultured as recommended using Dulbecco IS-OV (Sigma) containing 10% newborn calf serum (HyClone) and 40 $\mu\text{g}/\text{mL}$ gentamicin at 37 °C in a 5% CO₂ atmosphere.³¹ The drug-resistant variants of MCF-7 cell lines were previously established by stepwise selection after prolonged (>6 mo) treatment of original MCF-7 cells with escalating concentrations of DOX or CIS at a range of 0.5 to 15 $\mu\text{g}/\text{mL}$ in the medium. After 6 mo of culturing in the presence of drugs, the IC₅₀ (inhibitory concentration to produce 50% cell death) values were 19.2 and 3.6 mg/L for the MCF-7/DOX and MCF-7/CIS cells, respectively.

Cells were seeded at a density of 0.5×10^6 viable cells per 100-mm plate, and the medium was changed every other day for 6 d. At 70% confluency, the cells were treated with the studied antioxidant compounds at concentrations 350 $\mu\text{g}/\text{mL}$ for 2 h. Cell survival and morphology was evaluated under the microscope after 2 h of treatment.

RNA extraction and gene expression. Upon treatment, cells and tissues were flash-frozen in liquid nitrogen for storage and further analysis. Then, cells and tissues were homogenized in TRIzol® Reagent (Invitrogen). RNA isolation was performed according to manufacturer's instructions (Invitrogen). The RNA was quantified and the quality was found to be of a high quality using a Bioanalyser 2100 (Agilent). RNA labeling and microarray hybridization were performed by the Lethbridge Epigenetics Laboratory Illumina Facility. HumanHT-12 v4 Expression BeadChip whole-genome expression arrays (Illumina) were used in this study. Each array on the HumanHT-12 v4 Expression BeadChip targets more than 47 000 probes derived from the National Center for Biotechnology Information Reference Sequence (NCBI) RefSeq Release 38 and other sources (Illumina). Three biological replicates were used per each experimental group. In brief, each RNA sample was amplified using the Ambion Illumina RNA amplification kit

with biotin UTP (Enzo) labeling. The Ambion Illumina RNA amplification kit uses T7 oligo(dT) primer to generate single stranded cDNA followed by a second strand synthesis to generate double-stranded cDNA, which is then column purified. In vitro transcription was conducted to synthesize biotin-labeled cRNA using T7 RNA polymerase. The cRNA was column purified and checked for size and yield. cRNA was hybridized using standard Illumina protocols with streptavidin-Cy3 (Amersham). Arrays were scanned on an Illumina Beadstation and analyzed using BeadStudio (Illumina) as recommended.³⁸ Normalization, clustering, and significance analysis were done by the Lethbridge Epigenetics Laboratory Illumina Facility as previously described.³⁸

Protein extraction and western blotting. Protein extraction and western blotting was conducted as described.³⁹ Membranes were incubated with antibodies against MRE11 and PCNA (1:1000; Santa Cruz Biotechnology), cleaved caspase, γH2AX , p21 (1:1000; Cell Signaling). Antibody binding was revealed by incubation with horseradish peroxidase-conjugated secondary antibodies and the ECL Plus™ immunoblotting detection system (GE Healthcare). Chemiluminescence was detected by Biomax MR film (Eastman Kodak) and scanned, and by the FluoChemHD2 system (ProteinSimple). Protein loading was visualized by Coomassie brilliant blue R250 staining (BioRad, Mississauga).⁴⁰

The Annexin V assay. For apoptosis analysis, we used the Annexin V-FITC Apoptosis Detection Kit I (BD Biosciences) according to the manufacturer's protocol as described earlier. Cells were grown on 75 cm² cell culture flasks and treated with **2a** and **2b** compounds as previously described (see section on tissues and Treatments). The analysis was performed 2 h after treatment. In brief, cells were harvested, washed with PBS, resuspended in 1× binding buffer, stained with Annexin V and propidium iodide (PI) for 15 min at 25 °C in the dark and analyzed by flow cytometry using the BD FACS Canto flow cytometer. The results were represented as the percentage of gated Annexin V positive cells.⁴¹

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/cbt/article/25935

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