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Abbreviations:

SDG, secoisolariciresinol diglucoside;

HPLC-MS/ESI, high performance liquid chromatography-mass spectrometry /electron spray ionization.

ABSTRACT

Our previous study demonstrated that lignan metabolites enterolactone and enterodiol inhibited colonic cancer cell growth by inducing cell cycle arrest and apoptosis. However, the dietary lignans are naturally present as glycoside precursors such as secoisolariciresinol diglucoside (SDG) that has not been evaluated yet. This study is thus to test a hypothesis that the dietary SDG might have a different impact from its metabolites in human colonic SW480 cancer cells. Treatment with SDG at 0-40 μM up to 48 hrs resulted in a dose- and time-dependent decrease in cell numbers, which was comparable to enterolactone. The cell growth inhibited by SDG appeared not to be mediated by cytotoxicity, but cytostatic mechanism with an increase of cyclin A expression. Furthermore, HPLC analysis indicated SDG in the media was much more stable than enterolactone (95% of SDG survival vs. 57% of enterolactone after 48-hr treatment). When the cells were treated with either enterolactone or SDG at 40 μM for 48 hrs, the intracellular levels of enterolactone as measured by HPLC-MS/ESI were about 8.3×10^{-8} nmol/cell, but intracellular SDG or potential metabolites were undetectable. Taken together, SDG demonstrated similar effects on cell growth, cytotoxicity, and cell cycle arrest when compared with its metabolite enterolactone. However, the reliable stability and undetectable intracellular SDG in treated cells may suggest that a metabolism of SDG, if exposed directly to the colonic cells, could be different from the known degradation by microorganisms in human gut.

Keywords: Lignans, secoisolariciresinol diglucoside, enterolactone, cancer prevention, SW480 cells

1. Introduction

Potential cancer prevention by dietary lignan glycoside, i.e., secoisolariciresinol diglucoside (SDG), has been suggested to be mediated through its metabolites, enterolactone and enterodiol [1-5]. The chemical structures of SDG and its mammalian metabolite enterolactone are shown in Figure 1. Enterolactone and enterodiol are anaerobically formed via colonic bacterial fermentation [6-7]. Besides the bacterial fermentation, enterolactone and enterodiol can also be formed by other cell organelles such as a study that showed liver microsomes could generate lignan aglycones [8].

Many studies show that SDG has cancer preventive properties in animal models. Our previous study demonstrated that SDG levels in wheat bran from various wheat cultivars were correlated to antitumor activities in APC-Min mice [9]. We also found that dietary SDG at 0.01% significantly reduced azoxymethane-induced formation of aberrant crypt foci in F344 rats [10]. In addition, we have shown that enterolactone and enterodiol or both in combinations inhibit human cancer cell growth through inducing S-phase cell cycle arrest and apoptosis [9]. Experimental animal studies by others show that dietary supplementation with 73-293 μmol/kg SDG inhibits experimental metastasis of B16BL6 murine melanoma cells in C57BL/6 mice [11]. However, no *in vitro* studies have been done to specify cancer preventive property of SDG, although SDG metabolites, enterolactone and enterodiol, have been intensively investigated [12-18]. Therefore, we hypothesized that the dietary SDG might have a different impact from its metabolites in human colonic SW480 cancer cells.

The objective in this study is to determine the cancer preventive effects of SDG in human colon cancer SW480 cells. The effects of SDG on cellular growth and the underlying mechanisms regarding cytotoxicity and cell cycle interruption were studied and compared with

its metabolite enterolactone. SDG was also investigated for stability in the media when compared to enterolactone. Furthermore, HPLC- MS/ESI was used to evaluate whether SDG were taken up intracellularly into the cells, which may provide insight into its bioavailability and cancer preventive impact.

2. Methods and materials

2.1. Cell culture and treatments

The human colon cancer SW480 cells were purchased from the American Type Culture Collection (Rockville, MD), and cultured in the Dulbecco's Modified Eagle Media supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Sigma-Aldrich, St. Louis MO). The cells were cultured in either 6-well plates or 200 mL volumetric flasks at 37 ^{0}C in 5% CO_2 until 60-80% confluence. At this confluence, cells were treated with either SDG or enterolactone at 0-40 μM up to 48 hrs, at which the cells had reached less than 100% confluence. SDG was purchased from ChromaDex (Irvine, CA), while enterolactone was purchased from either Sigma-Aldrich (St Louis, Mo) or ChromaDex. SDG or enterolactone was dissolved in DMSO and then mixed with fresh media to achieve the final concentrations. DMSO was kept less than 0.2% in the final treatment, which did not alter cell growth or cell cycle measurements when compared with DMSO-free media (data not shown). After treatment, the cells were detached and counted by hemacytometer as described by our previous publication [9].

2.2. Cytotoxicity assay

The cell viability was measured in the adherent cells by trypan blue staining [9]. The viable cell numbers in the treated cells were compared with that in the vehicle controls.

2.3. Cell cycle analysis

The cell cycle analysis was done as previously described [9-10]. Briefly, the cells were fixed in ethanol, centrifuged and then the pellet was resuspended in the phosphate buffer saline solution at pH 7.4, containing 20 g/L propidium iodide and 5000 U/L of RNase (Promega) at 37 ^{o}C for 30 min. DNA flow cytometric analysis (FACSCaliber, Becton Dickinson, Newyork, NJ) was performed with an excitation at 488 nm and an emission at 630 nm.

2.4. Western blot analysis for cyclin A protein

As described in our previous publication [9], the treated cells were harvested and suspended in Triton lysis buffer. Protein concentration was measured by the Pierce BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL), and 30 μg of whole cell protein was electrophoresed on 12% SDS polyacrylamide gels and transferred to pure nitrocellulose membrane using semi-dry transfer cell (Trans-blot SD cell, BIO-RAD, Hercules, CA). The membrane was blocked in TDN buffer at 4 ºC for 1 hr. Then the membrane was incubated overnight with 1:1,000 dilution of anti-cyclin A and anti-β-actin (Cell Signaling Technology Inc., Danvers, MA). After washing, the membrane was incubated in anti-IgG of horse-radish peroxidase-conjugated secondary antibody (1:10,000) (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 hr. The specific protein band was visualized by applying the Super signal west femto maximum sensitivity substrate (Thermo Fisher Scientific, Rockford, IL). The band was scanned and detected by using FlouroChemTM 8800 Advanced Imagine System (Alpha Innotech, San Leandro, CA). The cyclin A protein levels at 55-kDa were normalized as ratios of the density of β-actin at 43-kDa in the same sample and then presented as the percentages of the vehicle controls.

2.5. HPLC quantification of SDG and enterolactone

HPLC-UV quantification of SDG and enterolactone was done according to previous methods [1, 9] with slight modification. Generally, the cell culture medium was separated by centrifugation at 1000 X g for 10 min from the treated cells after treatment with 0-40 μM SDG or enterolactone for 48 hrs. SDG and enterolactone were analyzed by HPLC using a C18 column (5 μm, 250 X 4.6 i.d. mm, Alltech, Deerfield, Il), eluted with a 5% acetonitrile in 0.01 mM phosphate buffer, pH 2.8 (solvent A) over 100% acetonitrile (solvent B) at a flow rate of 1 mL/min. A gradient runs at 0% solvent B for 10 min, 0-100% solvent B for 20 min, and 100% solvent B for 10 min. Peaks were detected by UV-monitoring at 283 nm. The enterolactone and SDG peak was identified according to both retention time and spectrum by comparison with a commercial standard, respectively. A linear HPLC calibration curve for both lignans was obtained for the concentrations between 0 and100 μM.

2.6. HPLC-MS/ESI analysis of intracellular levels of SDG and enterolactone

The SW480 cells treated with 40 μM SDG or enterolactone for 48 hr were harvested by detaching with trypsin-EDTA as previously described [9] and centrifuged at 1000 X g for 10 min. The supernatant was discarded and the pellet was washed by three times and resuspended in 0.01 M phosphate buffered saline at pH 7.4. The resulting solution was homogenized under an ice bucket followed by three 1:1 diethyl ether extractions. The upper organic layer containing the lignans was evaporated to dryness in a water bath set at 35 ºC under vacuum condition. The residue remaining at the bottom of the flask was re-dissolved in 100% methanol and ultra-centrifuged at 40,000 X g for 30 minutes. HPLC-MS/ESI analysis was performed with an Esquire 3000 plus mass spectrometer (Bruker Daltonics, GmbH, Billerica, MA) coupled to an Agilent (Agilent, Waldbronn, Germany) 1100 series HPLC system. Separations were achieved with a Synergi Max-RP (Phenomenex, Torrance, CA) column (20 x 2 mm i.d., 2 μm) with a

linear 2.5 min. gradient from 30-95% acetonitrile in water containing 0.1% formic acid. The MS/ESI spectra were acquired in positive ionization mode from *m*/*z* 100 to1000. The mass spectrometry instrument was controlled by the esquire control 5.3 software (Bruker Daltonics, GmbH, Billerica, MA). The data were processed with Data analysis 3.3 software (Bruker Daltonics, GmbH, Billerica, MA).

2.7. Statistical analyses

All data was analyzed by the SAS statistical system, version 8.2. The cell number, cell cycle and HPLC data were analyzed by 2-way ANOVA using a general linear model procedure followed by Fisher's protected least square difference. The relationship between lignan concentrations remaining in cell culture media and the original lignan amounts was determined by linear regression by using the Pearson's correlation coefficients (r). The results are present as means ± SEM and a probability at < 0.05 is considered significantly.

3. Results

3.1. Cell growth inhibition

As shown in Figure 2, treatment of SW480 cells with either SDG or enterolactone at 0-40 μM up to 48 hrs resulted in a dose- and time-dependent decrease in cell number when compared with the vehicle control.

3.2. Cytotoxicity Assay

The cell viability was generally greater than 80% in the adherent cells, and the treated cells did not differ significantly from the vehicle-treated controls (data not shown).

3.3. Cell cycle Analysis

The treatment of SW480 cells with either SDG or enterolactone at 30-40 μM for either 24 or 48 hrs induced cell cycle arrest at S-phase significantly (Figure 3). As the percentage of cells

149 in S-phase increased, the percentage of cells at both G1 and G2/M phases decreased
150 correspondingly.
151 *3.4. Cyclin A protein levels*
152 The levels of cyclin A protein significantly increased in the cells treated with SDG at 20-
153 40 μM or enterolactone at 40 μM for 48 hrs when compared to the vehicle control (Figure 4).
154 *3.5. Stability of SDG vs. enterolactone in the medium*
155 The concentrations of SDG and enterolactone in the cell culture media after treatment
156 were measured over the time by HPLC method. Figure 5A showed a representative HPLC
157 chromatograph of the standard SDG with two common SDG metabolites, i.e., enterodiol and
158 enterolactone. The retention times of SDG, enterodiol, and enterolactone are 20.5, 25.4, and 28.6
159 min, respectively. By using this established HPLC method, either enterolactone or SDG was
160 detectable in the media after treatment as shown in Figure 5B and 5C, respectively.
161 The stability of SDG and enterolactone in the media with or without SW480 cells for 48
162 hr was further evaluated. As shown in Figure 6, SDG generally exhibited greater stability than
163 enterolactone in the media with or without the cells. About 95% of SDG at 40 μM in the media
164 without the cells was detectable after 48 hr treatment verse 57% of enterolactone only. Both
165 SDG and enterolactone at 10-40 μM resulted in the greater losses in the presence of SW480
166 cells, likely due to uptake and/or degradation by cellular enzymes.
167 3.*6. Intracellular levels of SDG verse enterolactone*
168 In comparison with the representative HPLC chromatograph of SDG, enterodiol,
169 enterolactone, and internal standard flavone (Figure 7A), a HPLC chromatograph in Figure 7B
170 demonstrated a detectable enterolactone from the intracellular extracts of total 210 million cells

after treatment with enterolactone at 40 μM for 48 hr. However, SDG was not detectable from the intracellular extracts of the treated cells under a similar condition as shown in Figure 7C.

HPLC detection for both SDG and enterolactone peaks was further identified by MS/ESI analysis. The representative MS spectra were shown in Figure 8. SDG has a molecular mass at 687 and thus was shown in *m/z* of 687.02. Furthermore, the ion peak at *m/z* of 704.09 could be interpreted as a combination of SDG with a molecule of water $[SDG+H_2O]^+$, and the ion peak at *m/z* of 709.08 is most likely the sodium salt of SDG $[SDG+Na]^+$. The major ion identified as enterolactone had a mass/charge ratio of *m/z* at 298.46 (Figure 8B).

4. Discussion

Lignans are a group of the phytochemicals that compose of phenylpropane dimer linked by β-β bonds with a 1,4-diarylbutane structure, which are present abundantly in flaxseed, but also present in various grains such as wheat [9-10]. The most prominent lignan in dietary grains is SDG. When ingested, SDG undergoes bacterial fermentation to generate lignan mammalian metabolites, i.e., enterodiol and enterolactone [10]. Although both enterodiol and enterolactone have been shown for cancer preventive activities, little information is available about the potential cancer preventive property of SDG by itself. The absorption of mammalian lignans is usually poor, resulting in a low range of nM up to a few μM in the human plasma and/or urine samples [19]. However, lignan precursors-enriched foods may reach to the gut and provide a much high dose into the colonic contents. Bach Knudsen et al. once reported that the mammalian lignans could be detected up to 175 μmol/kg fecal samples in the stool of pigs fed rye based diets containing total lignans at 137 μmol/d [20], indicating that a much high dose of dietary lignans could be physiologically achieved in the colonic contents. Although the physiological significance of such high doses of lignans presented in the colonic contents is not defined, we

194 hypothesized that dietary SDG, in addition to be a precursor of enterodiol and enterolactone,
195 might influence the colonic mucosal cells *in situ*. Furthermore, a rational plan for using the
196 concentrations up to 40 μM tested in this study was also supported.
197 In this study, we demonstrate that SDG inhibits colon cancer cell growth by inducing S-
198 phase cell cycle arrest, a characteristic previously attributable mostly to its metabolites. In
199 addition, not only does SDG inhibit colon cancer growth but it is also much more stable than
200 enterolactone in the cell culture media.
201 When we reject our hypothesis that SDG might provide different effect on cancer cell
202 growth when compared with its metabolite entrolantone, we found the effects of SDG and
203 enterolactone on the cell growth, cell viability, and cell cycle arrest are comparable. However, a
204 higher stability of SDG in the media than enterolactone may be related to the nature of chemical
205 structure and the chemical groups attached. The bulky glucose moieties in the SDG structure
206 may prevent attack from the possible electrophiles in the cell culture media. In addition, the
207 presence of two methoxy groups attached to outer carbon benzene rings in the SDG structure
208 may allow for an increased electron delocalization, and hence could be more stable in the
209 inherent SDG structure [21]. This notion is consistent with the observation that SDG was stable
210 in the bread products during baking [22]. On the other hand, the reactive lactone group on
211 enterolactone tends to be unstable as reported in antibacterial studies by using lactone functional
212 groups [23, 24]. In addition, the accessibility of enterolactone functional groups to the potential
213 electrophiles appears high. Although, ChemDraw ultra software predicts a negative overall
214 enthalpy on the enterolactone structure [25], the lactone functional group and the openness of the
215 enterolactone structure may tend to overshadow this effect towards increased enterolactone
216 instability.

It is a challenge to detect intracellular levels of a phytochemical such as lignans. Based upon our previous experience in flavonoid apigenin [26] and lignan analysis [9-10, 27], HPLC method is usually sensitive at nanograms per million cells. By collecting over 210 million cells from a total of 21 cell culture flasks, the intracellular levels of enterolactone in enterolactone-treated cells were indeed measurable. HPLC-MS/ESI further confirmed enterolactone as a molecular ion with *m/z* at 298.47. However, the intracellular levels of SDG were not detectable, nor the common metabolites enterodiol and enterolactone, in SDG-treated cells. Some novel metabolites were revealed by MS/ESI, but we limited to identify the chemical structures for those potential metabolites in this moment. Although SDG and/or its metabolites were not detectable in the treated cells, it should be noted that it was not expected for intact SDG to be absorbed, since a phytochemical glycoside is usually deglycosilation by β-glucosidase or lactase phlorozin hydrolase [28]. Therefore, SDG-treated cells with undetectable intracellular SDG or its currently known metabolites observed in this study may provide novel insight into SDG metabolism and bioavailability if exposed directly to the colonic cells before degradation by microorganisms in human gut.

In conclusion, SDG displayed significant anticancer effects as indicated by the inhibition of colon cancer cell growth. This effect was associated partly with the induction of cell cycle arrest at S-phase. Also, the stability of SDG is much higher in the cell culture media than enterolactone. Intracellular level studies revealed that enterolactone taken up by enterolactone-treated cells, but not SDG as it was undetectable in SDG-treated cells. The reliable stability and undetectable intracellular SDG in treated cells may suggest a metabolism of SDG, if exposed directly to the colonic cells, could be different from the currently known degradation by microorganisms in human gut.

Acknowledgments

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Figure Legends

Figure 1. Chemical structures of the secoisolariciresinol diglucoside (SDG) verse its mammalian metabolite enterolactone.

Figure 2. Comparison of treatment with SDG verse enterolactone in SW480 cells for growth inhibition. The cells were co-cultured with either SDG or enterolactone at 0-40 μM for 24-48 hrs. The cell number was counted by hemacytometer. The results are present as Means ± SEM from 8-11 independent experiments. Means with different superscripts differ significantly, $P \leq 0.05$.

Figure 3. Comparison of treatment with SDG verse enterolactone for cell cycle arrest. The cells were co-cultured with either SDG or enterolactone at 0-40 μM for 24-48 hrs. The cell cycle analysis was performed by DNA flow cytometry. Results are present as Means ± SEM from 5-7 independent experiments for SDG treatment and 3-4 independent experiments for enterolactone treatment. Means with different superscripts differ significantly, $P \leq 0.05$.

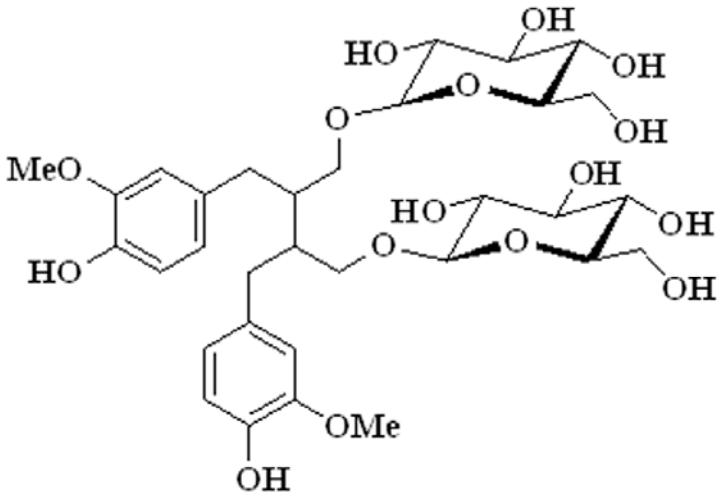
Figure 4. Effects of SDG verse enterolactone on the levels of cyclin A protein. The cells were co-cultured with either SDG or enterolactone at 0-40 μM for 48 hrs. The levels of cyclin A protein, as measured by Western blotting, significantly increased in the cells treated with SDG at 20-40 μM or enterolactone at 40 μM when compared to the vehicle control. Values are Mean ± SEM from 3 independent experiments. Means with different superscripts are significantly different, $P \leq 0.05$.

Figure 5. Representative HPLC chromatograph of SDG treatment verse enterolactone at 40 μM for 48 hrs in the cell culture media. Panel A: HPLC chromatograph of commercial standard SDG, enterodiol, and enterolactone; Panel B: HPLC chromatograph of enterolactone-treated medium; Panel C: HPLC chromatograph of SDG-treated medium.

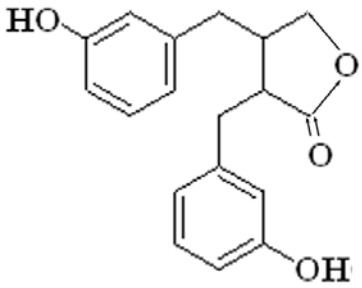
Figure 6. Stability of SDG verse enterolactone in the media with or without the cells. Either SDG or enterolactone was co-cultured at 40 μM in the media without cells or 0-40 μM with SW480 cells for 48 hrs. The levels of SDG or enterolactone in the medium extract with or without the cells were determined by HPLC method. Results are present as Means ± SEM from 3-4 independent experiments, *$P \leq 0.05$ vs. SDG treatment.

Figure 7. Representative HPLC chromatograph of SDG treatment verse enterolactone in the intracellular extracts. Panel A: HPLC chromatograph of commercial standard SDG, enterodiol, enterolactone, and internal standard flavone; Panel B: intracellular detection of enterolactone in 40 μM of enterolactone-treated cells for 48 hrs; Panel C: HPLC chromatograph of intracellular extracts in 40 μM of SDG-treated cells for 48 hrs.

Figure 8. HPLC-MS/ESI chromatograph of SDG (Panel A) and enterolactone (Panel B).



Secoisolariciresinol diglucoside (SDG)



Enterolactone

Fig. 1

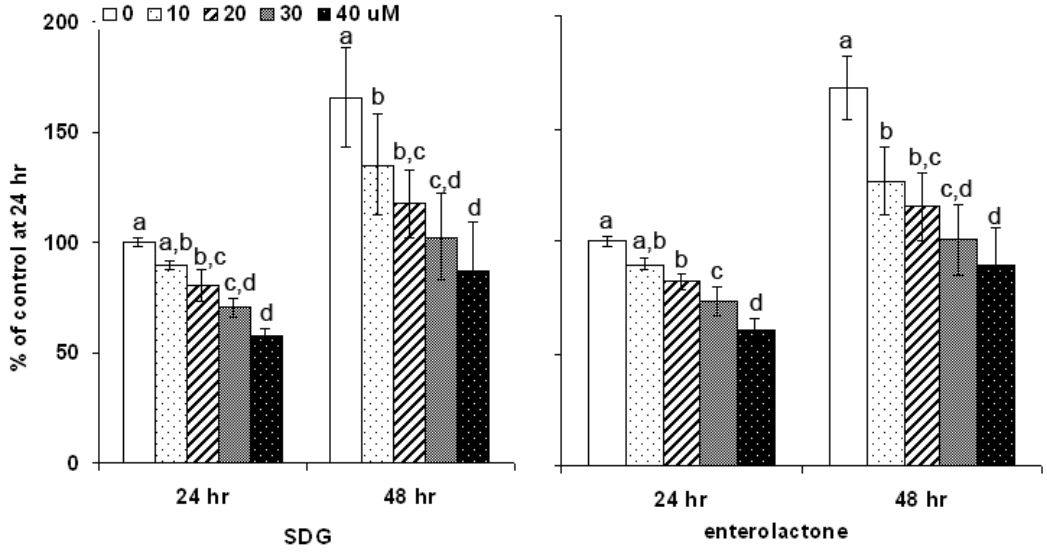


Fig. 2

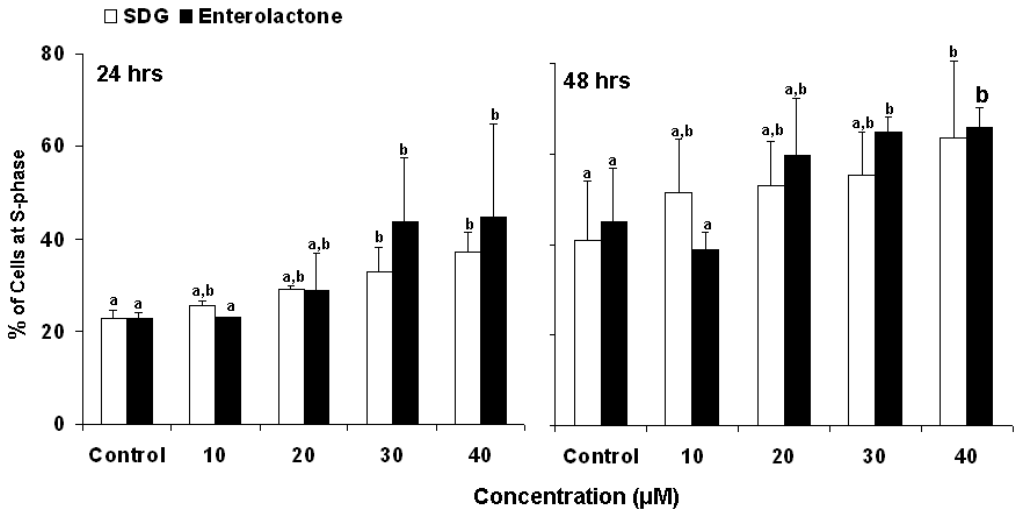
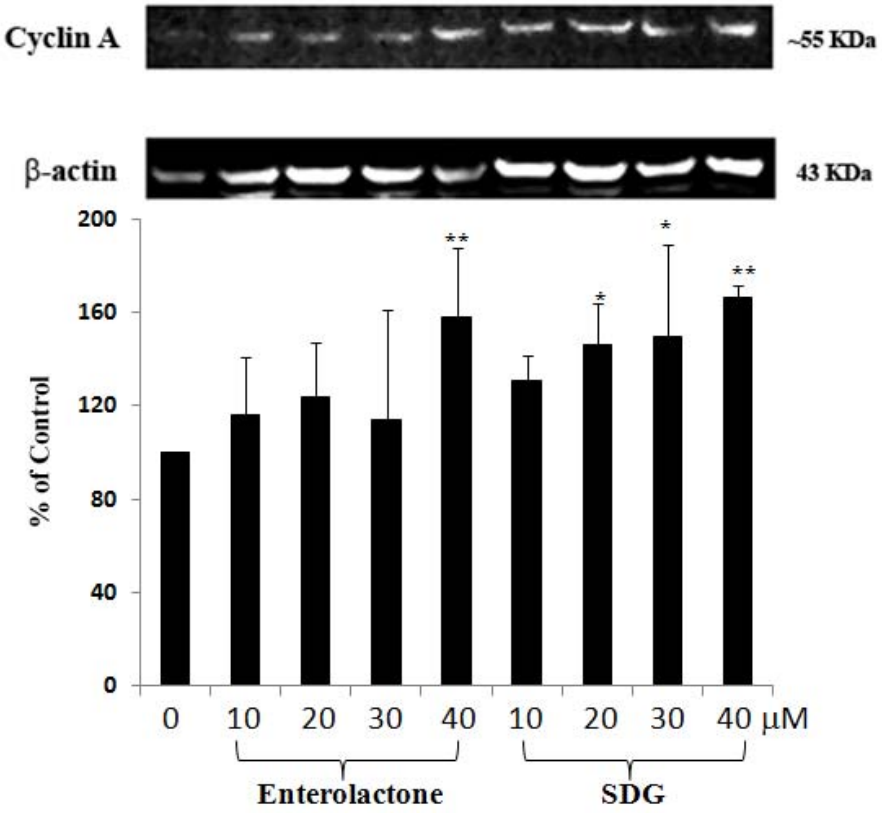

□ SDG ■ Enterolactone
24 hrs
48 hrs
% of Cells at S-phase
Control
10
20
30
40
Concentration (μM)

Fig. 3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~55 KDa
β-actin
43 KDa
% of Control
0 10 20 30 40 10 20 30 40 μM
Enterolactone
SDG

Fig. 4

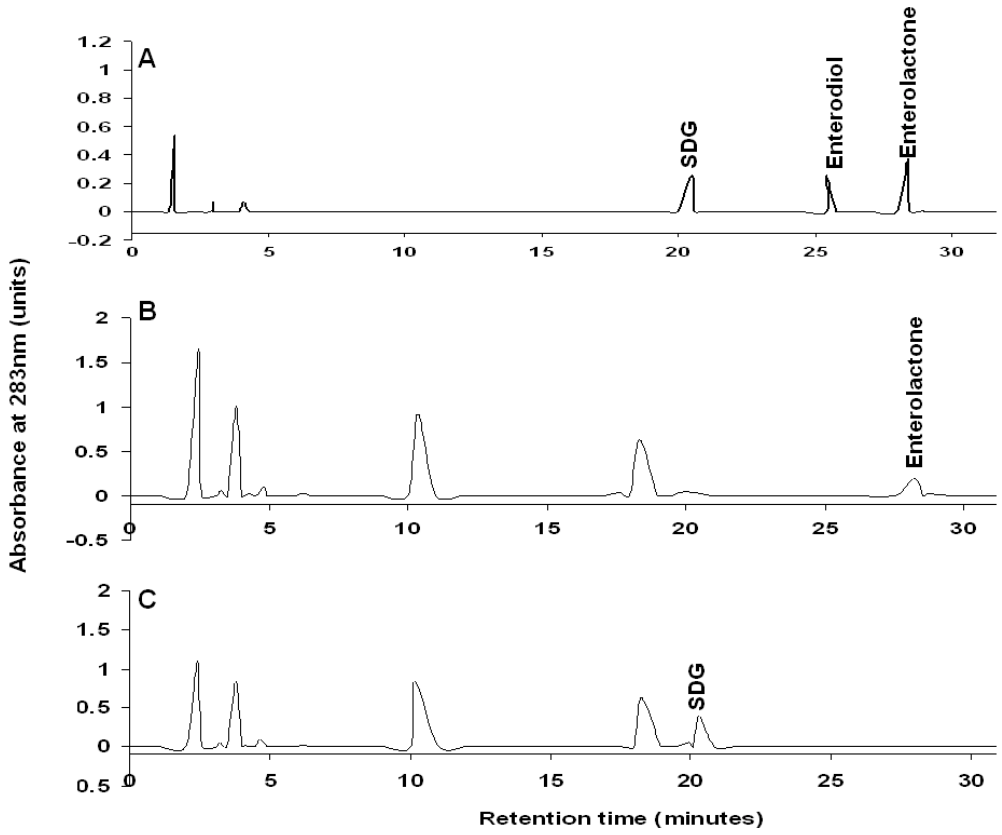
A
B
C
SDG
Enterodiol
Enterolactone
Enterolactone
SDG
Absorbance at 283nm (units)
Retention time (minutes)

Fig. 5

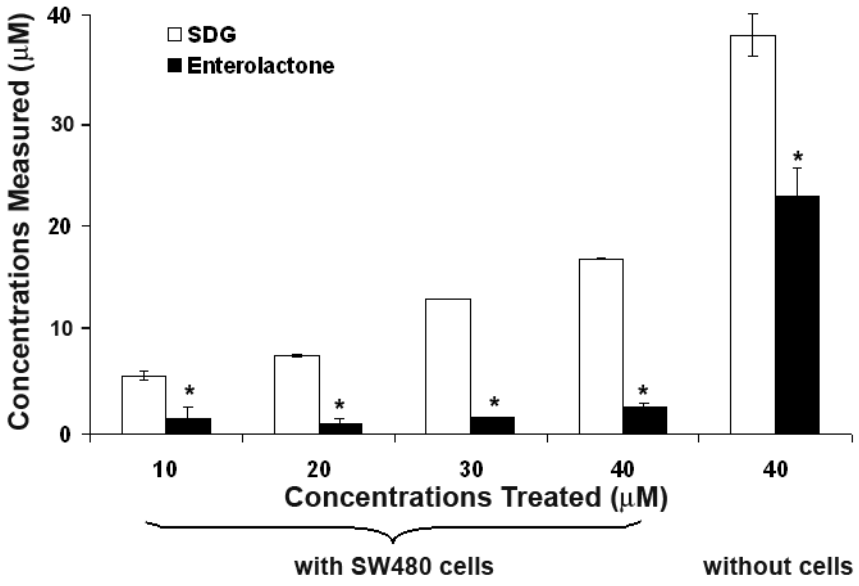
SDG
Enterolactone
Concentrations Measured (μM)
Concentrations Treated (μM)
with SW480 cells
without cells

Fig. 6

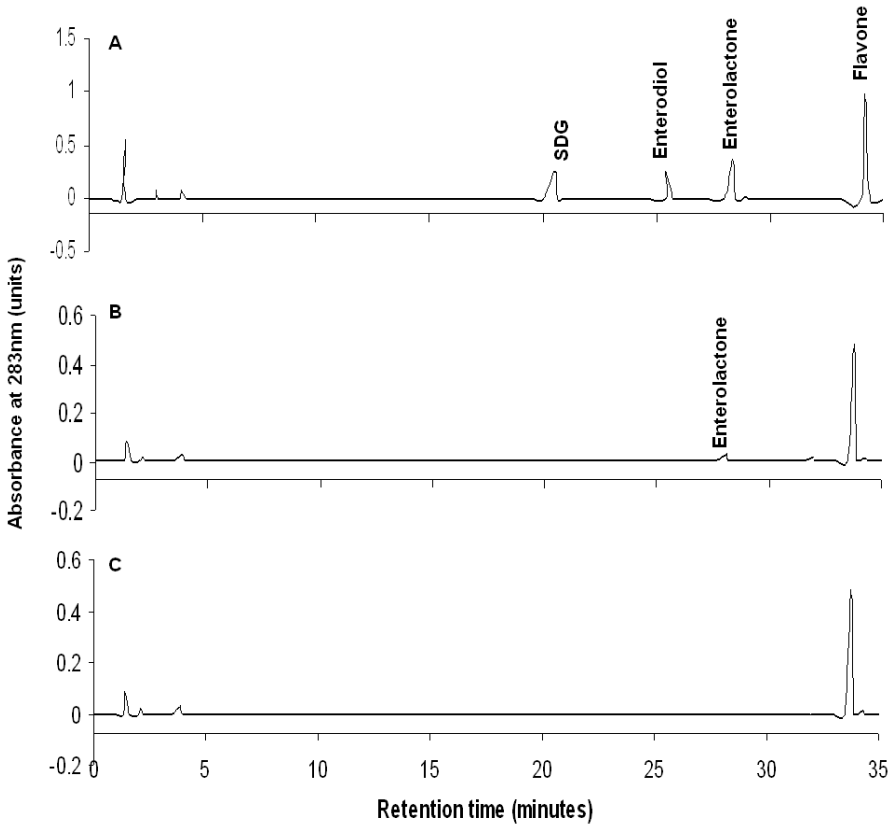
A
B
C
SDG
Enterodiol
Enterolactone
Flavone
Enterolactone
Absorbance at 283nm (units)
Retention time (minutes)

Fig. 7

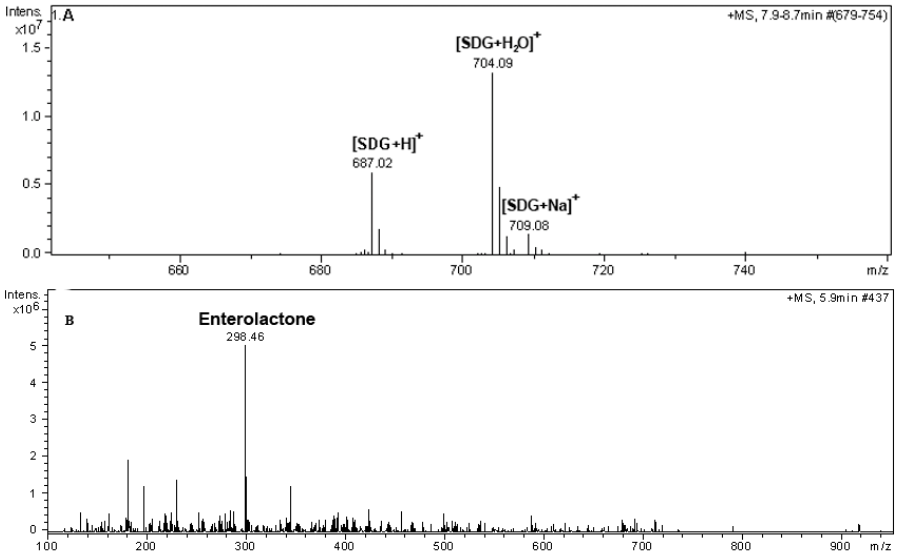
A
+MS, 7.9-8.7min #(679-754)
[SDG+H2O]+
704.09
[SDG+H]+
687.02
[SDG+Na]+
709.08
B
Enterolactone
298.46
+MS, 5.9min #437
m/z

Fig. 8