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BRIEF REPORT

Antiestrogenic and antiproliferative potency of secoisolariciresinol diglucoside derivatives on MCF-7 breast cancer cells

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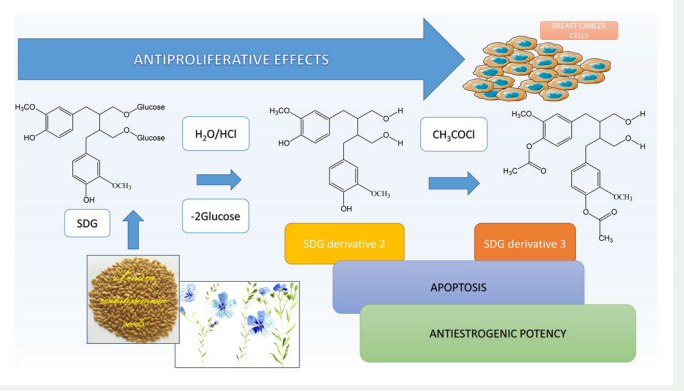
ABSTRACT

Secoisolariciresinol diglucoside (SDG) is isolated from *Linum usitatissimum* seeds. The antiproliferative effects of SDG **(1)** and its derivatives secoisolariciresinol **(2)** and secoisolariciresinol-4′, 4″-diacetate (**3**) have been evaluated on MCF-7 breast cancer cells and normal breast epithelial line MCF-10A. Lignan **1** has not shown cytotoxic effects on MCF-7 cells, while derivatives **2** and **3** have inhibited cell growth with IC_{50} values of 25 and 11 μM, respectively. Estrogen receptor alpha is a key growth driver in MCF-7 cells. Compound **1** did not affect the activity of ERα, while derivatives **2** and **3** showed significant antiestrogenic effects. Compounds **2** and **3** caused apoptosis in the MCF-7 line, determined by the cleavage of PARP. SDG derivative **3** enhanced the effect of doxorubicin. SDG derivatives can be considered as promising agents that exhibit a combined antiestrogen and proapoptotic effect in hormone-dependent breast cancer cells.



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1. Introduction

Lignans are natural biologically active compounds that consist of two phenylpropane units joined by a β-β-bond (Gigliarelli et al. 2018; Zálešák et al. 2019). Lignans are an available chemical class for drug development. The antitumor and antioxidant properties of lignans and their derivatives have been previously described (Gigliarelli et al. 2018; Zálešák et al. 2019; Teodor et al. 2020; Zhu et al. 2020a, 2020b). However, to date, the exact molecular mechanisms of action of this chemical class have not been fully studied.

The evaluation of antiproliferative effects of natural lignan SDG and its structural analogues is essential for understanding the biochemical basis and mechanism of anticarcinogenic and antitumor effects of this class of lignans, also for study of structure-activity relationships. Considering that the presence of glucose moiety makes the SDG molecule more hydrophilic and this factor influenced greatly on the penetration of this molecule through the lipophilic membrane of the cells, we reduced this part, as it happens firstly during the metabolism of this lignan (Kitts et al. 1999). Further lipophilization of the molecule of secoisolariciresinol could contribute to its faster penetration through the cell membrane. Also, we expect the increase in antiestrogenic effect due to this chemical modification, because the structures of secoisolariciresinol and secoisolariciresinol-4′,4″-diacetate are more similar to estrogens than the structure of SDG. It was partly confirmed by SwissTargetPrediction tool for estimation of the most probable macromolecular targets (Daina et al. 2019).

Lignan secoisolariciresinol diglucoside **1** was isolated from *Linum usitatissimum* seeds by extraction with 50% aqueous ethanol, followed by chromatographic purification on Diaion HP-20 ion-exchange sorbent and C18 reversed-phase silica gel (Stasevich et al. 2009). The synthesis of secoisolariciresinol (**2**) was performed by acid hydrolysis of **1**, as described previously by us (Stasevich et al. 2009). Secoisolariciresinol-4′, 4″-diacetate **3** was obtained in the reaction between **2** and acetyl chloride (Stasevich et al. 2009) (Scheme S1).

Considering high activity of various natural compounds against the luminal type of breast cancers (Salehi et al. 2020; Zhu et al. 2020a) and their potent antitumor effects described by us in (Stasevich et al. 2010), we have analyzed the effect of the compounds on MCF-7 breast cancer cells and identified signaling pathways involved in the response to SDG derivatives **2** and **3**.

2. Results and discussion

Cytotoxic effects on MCF-7 breast cancer cells and MCF-10A epithelial cells

MCF-7 cells were treated with SDG **1** and its derivatives **2** and **3** for 72 hours. As shown in Figure S1A, SDG did not exert a cytotoxic effect on MCF-7 cells, while its derivatives caused inhibition of cell growth. The IC_{50} value of SDG derivative **2** on MCF-7 cells was $25.1 \pm 2.9\,\mu M$, whereas the IC_{50} value of compound **3** was $11.5 \pm 1.8\,\mu M$.

Evaluation of the potential toxicity of new compounds is important for *in vitro* studies. The MCF-10A cell line is an *in vitro* model of normal breast epithelium (Graminha

et al. 2020; Waleka et al. 2020). SDG and its derivatives did not exert a cytotoxic effect on MCF-10A cells at concentrations lower than 50 μM. Cytotoxicity of **2** and **3** was demonstrated only at elevated concentrations (Figure S1B) with the IC_{50} values of 88.6 ± 6.9 μM and 93.5 ± 8.5 μM, respectively. Thus, the treatment with compounds **2** and **3** unveiled their effective and selective action towards MCF-7 breast cancer cells.

Since SDG **1** did not show cytotoxic effects on tumor cells, further studies were performed with compounds **2** and **3** demonstrating high activities.

The effect of glycosylation could be the reason for the absence of cytotoxic effect of natural lignan SDG in comparison with secoisolariciresinol and secoisolariciresinol-4′,4′′-diacetate against MCF-10A cell line. The analogous results were presented by us earlier against B-cell lymphoblastoid human cancerous cell line Raji (Stasevich et al. 2010) and in the research with flavonoid quercetin. It was shown that quercetin demonstrated significant antiproliferative effect, while its glucoside was inactive in concentrations below 463,4 μM (Kwak et al. 2009; Razavi et al. 2009). Acetylation, therefore, was demonstrated as a powerful tool to increase the cytotoxicity of secoisolariciresinol. The same results were presented earlier with the acetylated form of quercetin (Dell'Albani et al. 2017). This effect may be due to increased lipophilicity in the acetylated molecules.

The alternative explanation of the antiproliferative effects of secoisolariciresinol and secoisolariciresinol-4′,4′′-diacetate arises from their structural similarity with estrogen. It was reported that estrogen significantly inhibits cell growth of the ERα-negative human monoblastic leukemia cell line U937 via cell cycle arrest (Shim et al. 2003).

Antiestrogen activity of SDG derivatives and apoptosis

Estrogen receptor alpha (ERα) is expressed in about 70% of breast cancers. This makes hormone therapy possible in most breast cancer patients. Considering that ERα is expressed at a high level in MCF-7 cells and is a key proliferation driver, we have analyzed the effects of **1**, **2**, and **3** on the activity of this factor. For ERE-Luc assay, cells are transfected with the plasmids containing the luciferase reporter gene controlled by the promoter with estrogen-responsive elements (ERE-Luc). Cells are treated with a physiological receptor ligand, 17β-estradiol, and novel compounds. Figure S2 shows that compounds **2** and **3** inhibit the 17β-estradiol-induced luciferase activity. In the assay, both SDG derivatives were less active than the antiestrogen fulvestrant. Thus, tested SDG derivatives showed antiestrogenic potency. The explanation of effects for secoisolariciresinol and secoisolariciresinol-4′,4′′-diacetate arises from their structural similarity with estrogens, this fact is partly confirmed by SwissTargetPrediction tool for estimation of the most probable macromolecular targets (Daina et al. 2019).

The current classification of estrogen receptor regulators includes two classes: SERM (Selective estrogen receptor modulators) and SERD (Selective estrogen receptor degraders). The "gold standard" for hormone therapy, tamoxifen, belongs to the SERM group. Tamoxifen can inhibit the activity of the estrogen receptor, but in some tissues, it exhibits agonistic activity. Despite the undoubted success of this drug in clinical practice over the past 40 years, its agonistic activity and other side effects are limiting factors. The SERD group, which includes a fulvestrant, does not have agonistic activity

and its action is focused on ERα degradation. By immunoblotting, we have evaluated the effect of compounds **2** and **3** on the ERα expression level. It turned out that the treatment with **2** and **3** leads to a significant reduction of ERα expression in MCF-7 cells. Considering the nature of the effect on the level of ERα, compounds **2** and **3** can be assigned to SERDs.

The effect of **2** and **3** on the apoptosis marker, PARP cleavage, was analyzed using immunoblotting. Figure S3 shows that compounds **2** and **3** cause dose-dependent PARP cleavage. Thus, apoptosis is the main type of cell death induced by compounds **2** and **3**.

Combination with doxorubicin

One of the important problems in chemotherapy is the occurrence of toxicity when using high doses of drugs. One of the possible approaches to overcome this problem is the search for potential chemo-sensitizers. We used a combination of doxorubicin and compound **3** in low doses to treat MCF-7 cells. Figure S4 shows a significant increase in the effect of doxorubicin combined with compound **3** (CI $<$ 1). Thus, the development of strategies of combining **3** with chemotherapeutics is promising. New intriguing data published by Arken (Arken 2019) indicate the great potential of lignans to overcome the resistance of tumors to doxorubicin. In Arken's work, the multidrug resistance reverse effect of schizandrol A, a lignan from *Schisandra chinensis*, was demonstrated with P-gp overexpressing drug-resistant K562/A02 cells. Similar trends are identified in (Laiolo et al. 2018) for analogues of the lignan pinoresinol.

Apoptosis plays a pivotal role in the pathogenesis of cancers and is linked to many cell signaling pathways. Lignan-mediated enhancement of apoptosis has been shown in several studies; it can occur through many mechanisms. For example, the data acquired by Li-Hua Chen et al. (Chen et al. 2007) show the inhibitory effect of enterolactone on LNCaP cell (human prostate carcinoma) growth. Treatment of LNCaP cells with 25 to 100 μM of enterolactone for 24 h resulted in the disruption of mitochondrial membrane potential and induction of mitochondrial-mediated cell death. Enterolactone also caused a dose-dependent release of cytochrome c and an increase in the cleavage of caspase-3 and PARP, events that are considered molecular hallmarks of apoptosis. Enterolactone-induced apoptosis may also be mediated through the inactivation of the Akt signaling pathway, as suggested by the reduced phosphorylation of Akt and its downstream targets (GSK3 and MDM2), and enhanced p53 expression. The similar trends were seen in the study (Buckner et al. 2019), where treatment of B16-BL6 murine melanoma and MCF-7 breast cancer cells with flaxseed oil induced apoptosis as determined by changes in cell morphology (increase in the sub-G1 population in treated cells), annexin V staining (associated with phosphatidylserine "flipping" to the outer leaflet of the plasma membrane), DNA fragmentation and/or caspase activation (activation of caspase-9 is consistent with activation of the intrinsic apoptotic pathway and results in activation of caspase-3; activation of caspase-8 is associated with the extrinsic apoptosis pathway that results from a receptor-mediated activation of an apoptotic signal in the plasma membrane).

As it was shown, lignans demonstrate weak binding properties to estrogen receptor α (ERα) and ERβ. The results of Stefan O. Mueller et al (Mueller et al. 2004) study show

that enterolactone and 6-OH-enterolactone were partial agonists/antagonists of ERα or ERβ.

Yunyun Di et al. (Di et al. 2018) have gathered evidence that flaxseed lignans, secoisolariciresinol and enterolactone, could enhance the anticancer activity of therapeutic agents against breast cancer cell lines. Secoisolariciresinol selectively enhanced the cytotoxicity of docetaxel in SKBR3 and MDA-MB-231 cells. Exposure with 50 mM enterolactone plus docetaxel gave the most stable combination effect and consistently resulted in very high DRI values of up to 10-fold in SKBR3 and MDA-MB-231 cells. Furthermore, enterolactone significantly increased the cytotoxicity of therapeutic concentrations of metformin in MDA-MB-231 cells in a concentration-dependent manner, at 50 mM increased the sensitivity of MDA-MB-231 cells to docetaxel by 10-fold and metformin by 2.3-fold. These data support the possibility of using flaxseed lignan supplementation as an adjuvant therapy to decrease cytotoxic chemotherapeutic drug dosage requirements while enhancing their effectiveness.

Lignans and other natural compounds can be used to overcome resistance to chemotherapy (Li et al. 2018; Morsy et al. 2018). In the 2020 work (Morsy et al. 2020), secoisolariciresinol alone showed dose-dependent cytotoxicity, where 25 or 50 μM of secoisolariciresinol caused significantly less viability of doxorubicin-resistant cells compared to control. When 50 μM secoisolariciresinol was added to doxorubicin, secoisolariciresinol significantly enhanced doxorubicin-induced cytotoxicity compared to doxorubicin alone. Morsy's et al results suggest secoisolariciresinol as a novel P-gp inhibitor that can sensitize cancer cells during doxorubicin therapy (Morsy et al. 2020). There are various natural inhibitors of P-gp to overcome doxorubicin-resistance in the resistant cell line (Morsy et al. 2018). In the 2018 work of the same research group, both in silico and in vitro studies confirm that piperine is an inhibitor of P-gp (Morsy et al. 2018).

In general, the effect of new derivatives of SDG on breast cancer cells and normal epithelium was analyzed. The tested compounds induced apoptosis in the MCF-7 line, determined by the PARP cleavage, and demonstrated selectivity for malignant cells. Antiproliferative effects of compound **3** are associated with inhibition of ERα, the main driver of growth of luminal breast cancer. Moreover, the secoisolariciresinol-4′, 4″-diacetate (derivative **3**) enhanced the effect of doxorubicin. New derivatives of SDG can be considered for further development, including for new approaches to therapy in combination with doxorubicin and other chemotherapeutic drugs. The combination of compound **3** with doxorubicin may be of interest for preclinical studies to lower the drug doses.

Disclosure statement

No potential conflict of interest was reported by the authors.

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