Interrogating the Estrogenic Activity of Sakuranetin, a Phytoalexin Extracted from Rice

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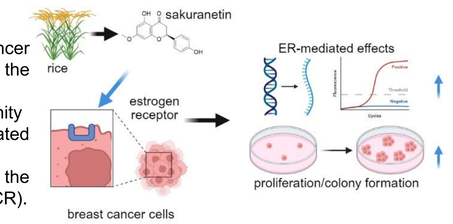
Sakuranetin's role as a phytoestrogen

Every year approximately 2.3 million people are diagnosed with breast cancer, of which around 685,000 will likely die. Breast cancer is classified by three major receptors: Estrogen Receptor (ER), Progesterone Receptor (PR), and Human Epidermal Growth Factor Receptor 2 (HER-2). Depending on the breast cancer subtype, these receptors can be present or not. ER+ cell lines require estrogen for cancer cell growth and are distinct from other subtypes in their response to therapy, aggressiveness, and growth. Flavonoids are a family of natural plant polyphenols subdivided based on the substituents on their aromatic rings. These compounds are detected in a wide variety of vegetables, legumes, and fruits. Of these, sakuranetin demonstrates promise as a bioactive natural compound. Many flavonoids have been designated as phytoestrogens based on their ability to interact with estrogen receptors (ER) to elicit strong anti-estrogenic and estrogenic responses. In recent years, these compounds have shown promising results that when ingested, led to decreased risk of breast cancer. Despite growing interest in natural compounds and the ER-agonist activity of sakuranetin precursors, the estrogenic effects of sakuranetin remain understudied. This study aims to evaluate the potential estrogenic activity of sakuranetin with the following objectives:

1. Assess estrogenic effects in breast cancer cell lines endogenously expressing the estrogen receptor
2. Measure sakuranetin – ER binding affinity
3. Evaluate sakuranetin-mediated proliferation and colony formation
4. Examine the impact of sakuranetin on the cellular transcriptome (RNAseq and PCR).



Background and Motivation

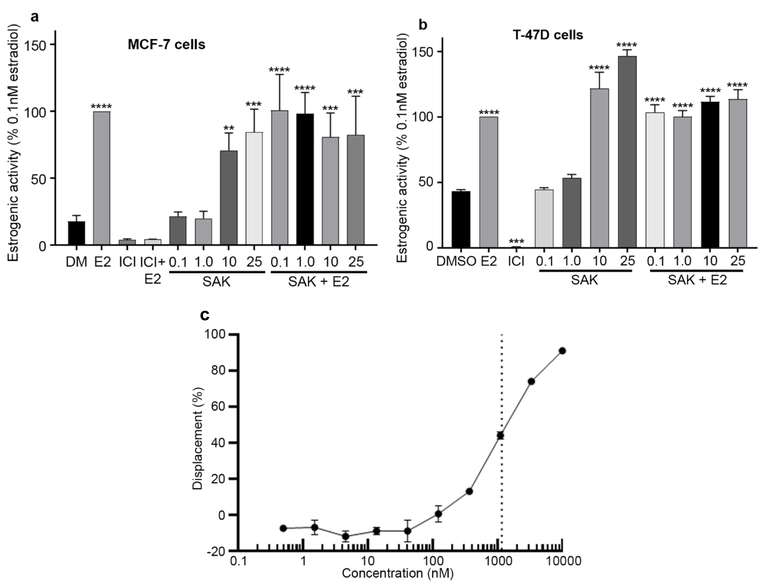


Figure 1. Sakuranetin elicits dose-dependent estrogenic responses in cell lines with endogenous ER. (a) MCF-7 or (b) T-47D cells were transiently transfected with pGL2-ERE2x-TK-luciferase plasmid for 6 h before treatment. Data represents mean ± SEM of 3 independent experiments. One-way ANOVA, Dunnett's multiple comparisons, compared to DMSO. **p<0.01, ***p<0.001, and ****p<0.0001. **Binding affinity of sakuranetin to the ER pocket requires greater than 1000 nM concentrations to initiate displacement**. (c) E2 was used as positive control, with an IC50 of 0.283 nM. Dotted line indicates calculated IC_{50} value, 1,170 nM. Data represents mean ± SEM of 2 independent experiments.

Sakuranetin Significantly Increases Proliferation and Colony Formation

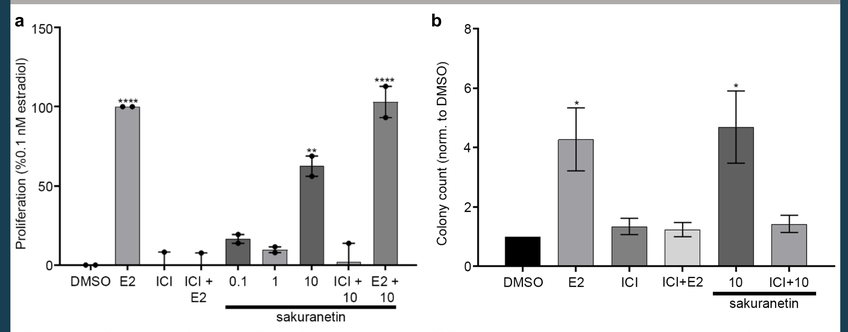


Figure 2. Sakuranetin significantly increases MCF-7 colony formation and proliferation while exhibiting no antagonist activity when combined with E2. (a) Cells were treated DMSO, E2 (0.1 nM), ICI (1 μM), sakuranetin (0.1, 1, or 10 μM), or combinations of sakuranetin and ICI (1 μM) or E2 (0.1 nM) for 24 h. Proliferation was evaluated using AlamarBlue and normalized to the positive control (0.1 nM E2). Data represents mean ± SEM of 3 independent experiments. (b) Prior to treatment, media was replaced with stripped media for 48 h, then treated with DMSO, E2 (0.1 nM), ICI 182 780 (0.1 μM), sakuranetin (10 μM), or combinations of sakuranetin and ICI (0.1 μM) or E2 (0.1 nM). At end point, cells were fixed, stained with crystal violet, and then counted. Data represents mean ± SEM of 3 independent experiments. One-way ANOVA, Dunnett's multiple comparisons test, compared to DMSO. *p<0.05.

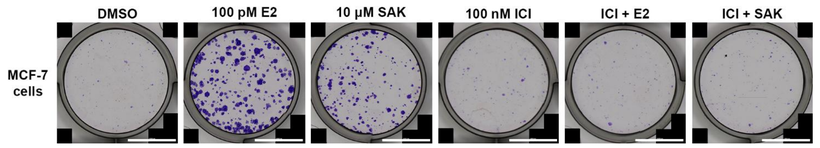


Figure 3. Representative images of MCF-7 colony formation. Prior to treatment, cell media was replaced with stripped media and treated with DMSO, E2 (0.1 nM), ICI (0.1 μM), sakuranetin (10 μM), or combinations of sakuranetin and ICI (0.1 μM) or E2 (0.1 nM). At end point, cells were fixed, stained with crystal violet, and then counted. Scale bars are 10 mm.

Sakuranetin Alters Estrogenic Gene Expression

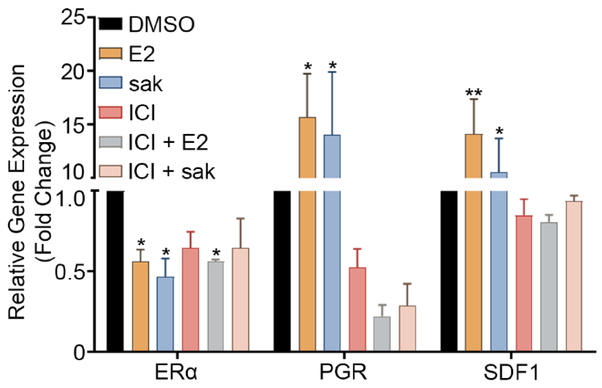


Figure 4. Sakuranetin induces estrogen response genes the ER+ cell line, MCF-7. Data was normalized to DMSO control and housekeeping gene (β-actin). qRT-PCR was performed for ERα and ER response genes, PGR and SDF1. Data represents mean ± SEM of 3 independent experiments. One-way ANOVA, Dunnett's multiple comparisons, compared to DMSO. *p<0.05 and **p<0.01.

Sakuranetin Induces An Estrogenic Transcriptomic Signature

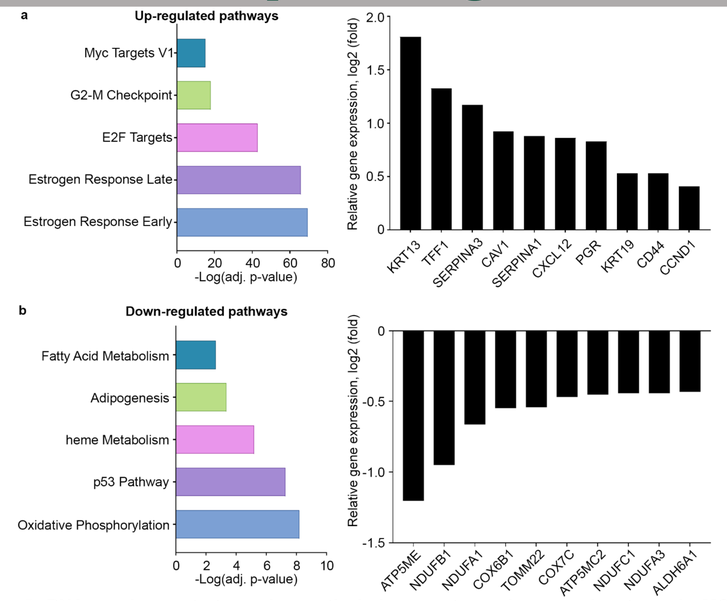


Figure 5. RNAseq demonstrates sakuranetin induces estrogenic gene signatures in MCF7 cells. MCF7s were treated with sakuranetin (10 μM) for 24 hours. Results demonstrate pathway analysis using Enrichr and the MSigDB Hallmark gene set database for **(a)** Top up-regulated and **(b)** Top down-regulated pathways with relative gene expression for estrogen-response genes significantly changed in treated cells. Data represents mean ± SEM of 3 independent experiments with comparisons to vehicle (DMSO).

Discussion & Future Works

Phytocompounds provide unique opportunities to enhance commercial crops or their byproducts through increased bioactivity. Our previous **major findings**:

- Sakuranetin-treated ER+ cell lines exhibited a dose-dependent response in estrogenic activity
- ER binding affinity was lower than synthetic E2, but similar to or higher than previously evaluated phytocompounds.

My work directly results in the following **major findings**:

- Sakuranetin significantly modulated ER+ cell proliferation and colony formation
- ER+ cells significantly increased the ER transcriptome and ER-mediated gene expression, after treatment

Future implications:

With the recent paradigm shift towards natural compound research, our findings suggest therapeutic application of sakuranetin, a novel underutilized phytocompound. Future studies will expand investigation of potential elicitors and plants that exhibit increased levels of sakuranetin.

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