Screening and characterization of pharmacologically active molecules that modulate embryo implantation using a in-vitro and in-vivo models

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Introduction

A receptive human endometrial environment is crucial for successful embryo implantation. The use of in vitro co-culture model allows us to study the effect of molecules on the attachment of spheroids (blastocyst surrogates) onto human endometrial epithelial cells (uterine epithelium surrogates). The library of pharmaceutically active compounds (LOPAC) containing 1280 pharmaceutically active molecules is commercially available for novel drug discovery. We aim to find out the non-hormonal molecules can effectively modulate embryo implantation and show the potential in the development of novel emergency contraception or medical abortion.

Hypothesis

We hypothesized that small molecules in the LOPAC library can modulate the human implantation process which show potentials in emergency contraception (EC) or medical abortion.

Objectives

1. To establish a high-throughput in-vitro implantation model for drug screening;
2. To screen small molecules from the LOPAC library that reduce spheroid attachment;
3. To evaluate the cytotoxicity of the top 20 small molecules that suppress spheroid attachment;
4. To study the attachment and cytotoxicity effect of selected molecules at lower concentrations;
5. To evaluate the attachment and cytotoxicity of selected molecules in other endometrial cell lines;
6. To confirm the in vivo effect of selected molecules on mouse embryo implantation.

Methodology & Results

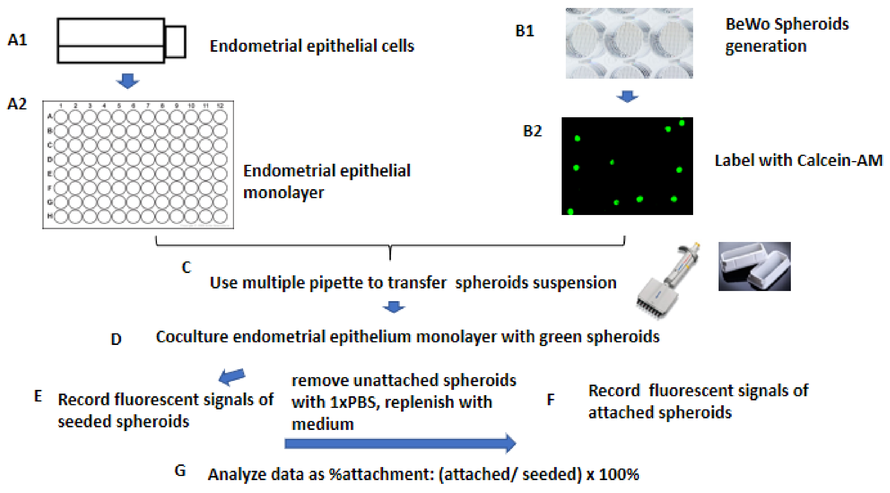

Figure 1 A schematic showing a high-throughput screening and co-culture assay. (A1) The endometrial epithelial Ishikawa cells were cultured in flasks and (A2) plated on a 96-well plate to form a monolayer. (B1) BeWo spheroids (blastocyst surrogates) were generated by AggreWell overnight and (B2) labeled with Calcein-AM (0.5μg/ml) for 30min. (C) About 75 spheroids were transferred on endometrial monolayer in each well and (D) coculture for 1 hour. (E) Fluorescence signals of seeded spheroids were measured by fluorescent plate reader. (F) The fluorescence signals of spheroids were measured again after removing the unattached ones. (G) The attachment rate was determined as percentage of attached to total spheroid added for co-culture.

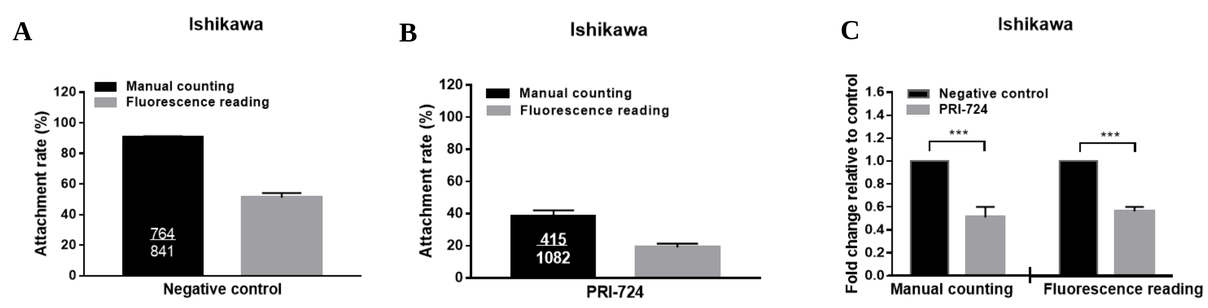

Figure 2 Comparison of manual counting and fluorescent detection of attached spheroid after co-culture. (A) The attachment rates of BeWo spheroids as determined by manual counting (dark bar) and fluorescence reading (gray bar). (B) The attachment rates of BeWo spheroids as determined by manual counting and fluorescence reading after treating the Ishikawa cells with PRI-724 10 μM for 24 hours. (C) Comparison of the fold change in the attachment rate with and without PRI-724 treatment between manual counting and fluorescence reading. Treatment group was compared with negative control by nonparametric unpaired *t* test, ***denotes significant difference (p<0.001) between groups (n=3).

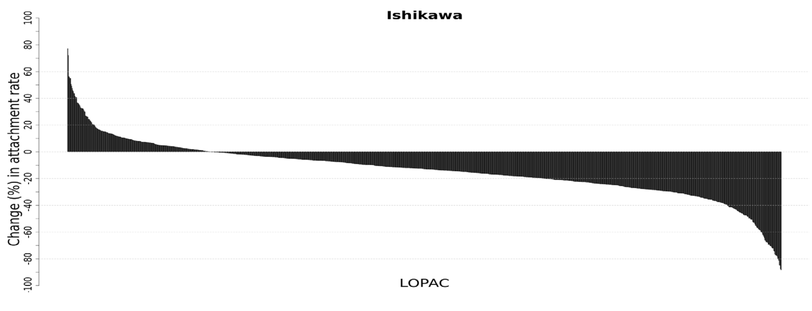

Figure 3 Coculture result of LOPAC library screening in the high-throughput endometrial-BeWo spheroids implantation model. (A) The Ishikawa cells were seeded on 96-well plate at $2.3x10^5$ cells/ well. After 24 hours, these cells were treated with and without LOPAC molecules at 10 μM, 0.1% DMSO and PRI-724 at 10 μM for 24 hours. The monolayers were cocultured with labeled spheroids and fluorescence signals were recorded as described above (Figure 1). Change in attachment rate = (attachment rate from treatment group - mean of attachment rate from negative control)/mean of attachment rate from negative control x 100) (n=2, in duplicates).

Molecule ID	Change (%) in Attachment Rate	Mean Difference	95.00% CI of Difference	Adjusted P Value
1	-88	43.25	26.96 to 59.54	0.0001
2	-88	43	28.98 to 57.02	0.0001
3	-85	42.21	30.55 to 53.88	0.0001
4	-85	36	20.76 to 51.24	0.0001
5	-81	40.88	26.48 to 55.27	0.0001
6	-80	32.88	18.18 to 47.57	0.0001
7	-80	27.5	11.07 to 43.93	0.0001
8	-78	39.71	28.05 to 51.38	0.0001
9	-78	32.88	16.11 to 49.64	0.0001
10	-77	33.13	20.97 to 45.28	0.0001
11	-77	32.75	17.51 to 47.99	0.0001
12	-77	33	15.24 to 50.76	0.0001
13	-74	26.75	10.89 to 42.61	0.0001
14	-74	31.75	16.51 to 46.99	0.0001
15	-72	30.63	13.86 to 47.39	0.0001
16	-72	35.25	21.92 to 48.58	0.0001
17	-72	29.88	17.6 to 42.15	0.0001
18	-71	29.88	17.6 to 42.15	0.0001
19	-70	35.46	23.8 to 47.13	0.0001
46	-55	29.88	13.11 to 46.64	0.0001

Table 1 Top 20 primary hits sorted on p-values and percentage changes. Raw coculture results from treatment groups were compared with negative control by nonparametric unpaired *t* test, a p-value less than 0.05 denotes significant difference between groups. These molecules were selected for cytotoxicity test and later experiments.

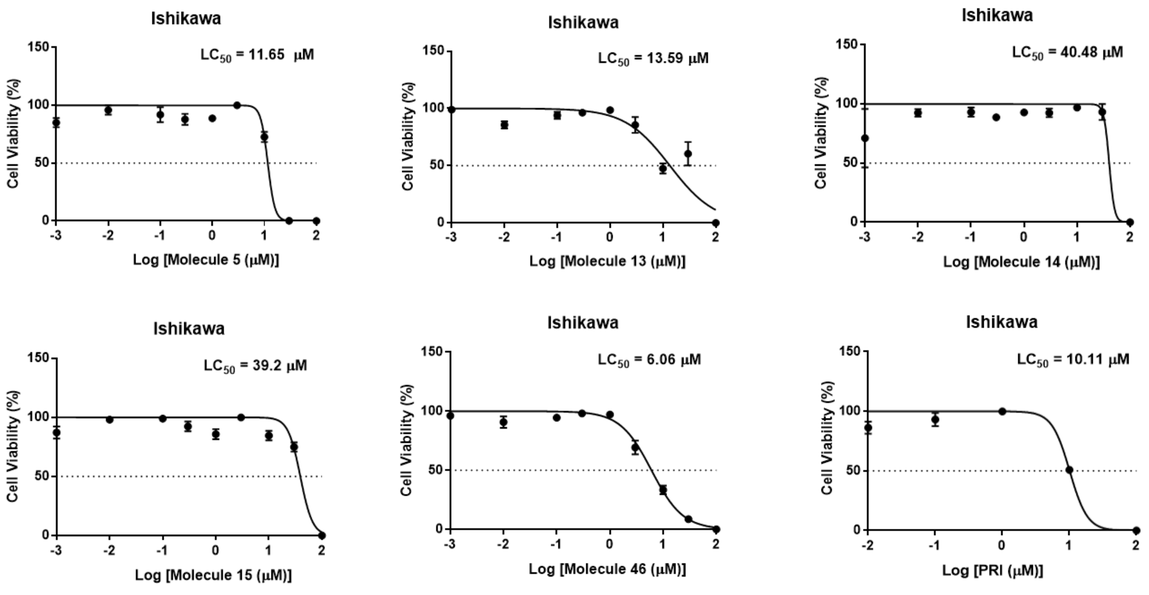

Figure 4 The dose-dependent effect of selected molecules on Ishikawa cell viability. Ishikawa cells were treated with and without different concentrations (0.01-100 μM) of **top 20 selected molecules (Table 1)** and PRI-724 for 24 hours before CyQUANT XTT cell viability test (n=3). The LC_{50} values of molecules (**5, 13, 14, 15 and 46**) and PRI-724 larger than their effective doses were shown on the above.

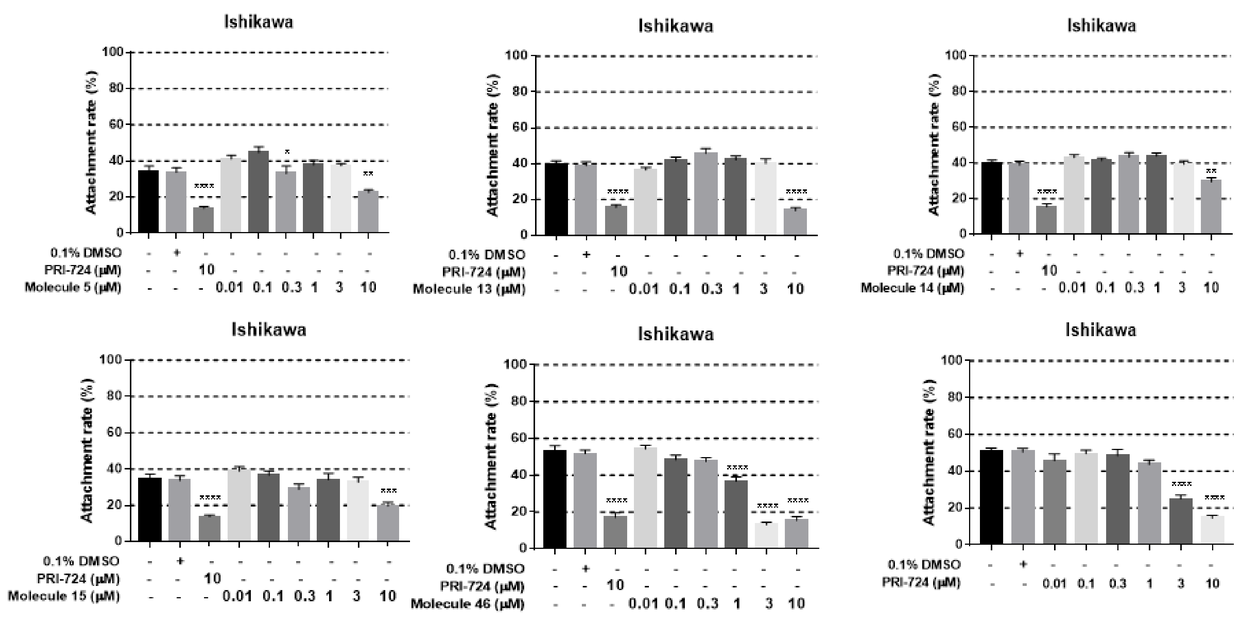

Figure 5 Dose-dependent effect of selected small molecules and PRI-724 on Ishikawa cell attachment. The Ishikawa cells were treated with and without different concentrations (0.01-100 μM) of selected molecules (**5, 13, 14, 15 and 46**) and PRI-724 for 24 hours before they were cocultured with BeWo spheroids (n=3). PRI-724 (10 μM) and 0.1% DMSO were used as positive control and vehicle control, respectively. (Kruskal-Wallis test, *p<0.05, **p<0.01, *** p<0.001, ****p<0.0001).

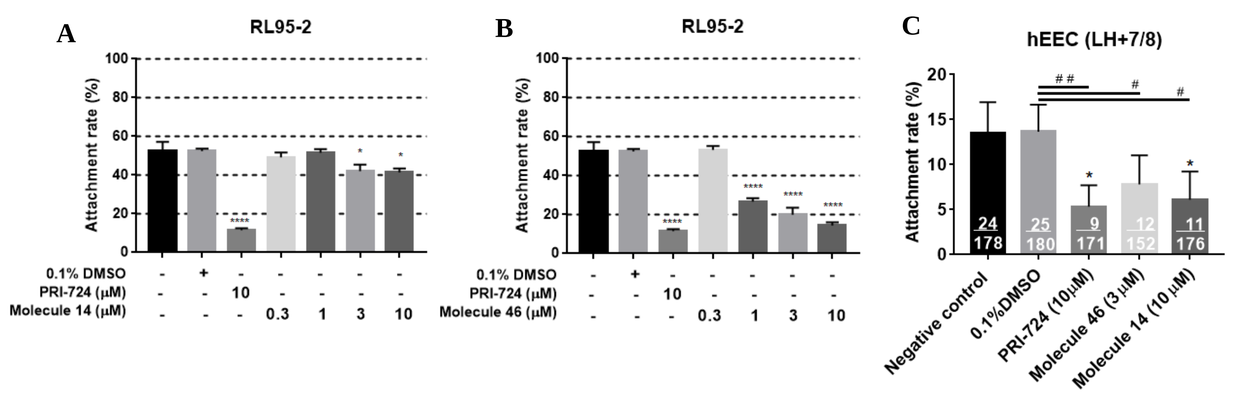

Figure 6 The attachment effect of Molecule 14 and 46 in RL95-2 and human primary endometrial epithelial cells (hEEC). (A & B) The RL95-2 cells were treated with and without selected molecules (**14 and 46**) at 0.3-10 μM 24 hours before they were cocultured with BeWo spheroids (n=3, Kruskal-Wallis test, *p<0.05, **p<0.01, *** p<0.001, ****p<0.0001). (C) The hEECs were treated with and without selected molecules (14 and 46) at 3 or 10 μM for 24 hours before they were cocultured with BeWo spheroids (n=7-8, Mann Whitney test, *p<0.05, **p<0.01, *** p<0.001, ****p<0.0001).

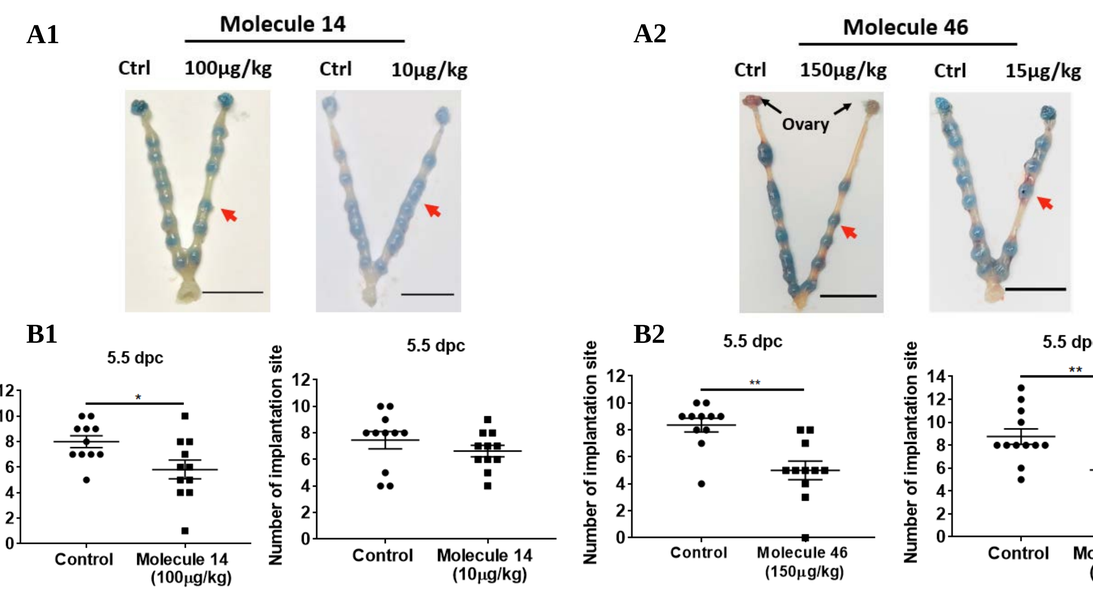

Figure 7 The effect of Molecule 14 and 46 on embryo implantation in the natural pregnant ICR mice model. (A) Representative figures of implantation number on 5.5dpc after cervical transfer of effect Molecule 14 (A1, 100μg/kg, 10μg/kg) and Molecule 46 (A2, 150μg/kg, 15μg/kg) on 1.5dpc (black arrow: ovary; red arrow: implantation site). (B) The number of implantation sites of the treated and control sides detected on 5.5dpc after the transcervical transfer of the molecules (Mean ± SEM, *p <0.05, **p<0.01 compared with the control, n=11-12).

Conclusions

1. **There was a total 175 (13.7%) small molecules found to significantly (p<0.05) suppress spheroid attachment from the LOPAC 1280.**
2. **Five small molecule were confirmed to have low cytotoxicity to Ishikawa cells, and suppress spheroid attachment at ≤10μM.**
3. **Molecules 14 and 46 suppress the attachment of BeWo spheroids on Ishikawa cells, RL-952 cells and LH+7/8 hEECs.**
4. **Molecules 14 and 46 can significantly decrease the number of implantation sites of treated side when comparing with the control of untreated side of the uterine horns in natural pregnant ICR female mice. These molecules could be potentially used to suppress embryo implantation in EC.**

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