Oocyte-Based Screening System for Anti-Microtubule Agents

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Abstract. Taxol and vinblastine have been widely used in cancer chemotherapy as anti-microtubule agents. However, there are on-going efforts to find new anti-microtubule agents with fewer of the side effects associated with these drugs, such as toxicity or the development of resistance. The standard method used to identify anti-microtubule agents has been the in vitro microtubule polymerization assay. One limitation of this system is that the only compounds selected are those that act on tubulin. Novel compounds whose targets are upstream or are related unknown molecules are not detected. Therefore, many researchers have recently tried to develop novel, phenotype-based drug screening systems. In this study, we developed an oocyte-based screening system for anti-microtubule agents. Dramatic phenotypic changes in microtubules can easily be observed in ovulated oocytes treated with microtubule-stabilizing or -destabilizing agents, such as taxol or vinblastine. After culturing with test samples for 5 h, oocytes were analyzed with fluorescence microscopy after immunostaining. In the oocyte-based screening system, the effective dose (ED50) of taxol for microtubule polymerization is ~5 nM, and the ED50 of vinblastine for microtubule depolymerization is ~2.5 nM. In addition, taxol-like and vinblastine-like compounds can be evaluated simultaneously in a single assay using this system.

Key words: Oocyte-based assay, Tubulin, Taxol, Vinblastine, Anti-microtubule

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have recently tried to develop novel, phenotype-based drug screening systems [12, 13].

Most mammalian ovulated oocytes are arrested in the metaphase of the second meiotic division unless activated by fertilization [14, 15]. These synchronized materials are very useful for the study of microtubule dynamics and the metaphase-to-interphase transition of the cell cycle. They are also convenient for the histochemical study of intracellular structures because of their large spindle apparatuses and number of microtubule organizing centers (MTOCs) [16]. Oocytes exhibit dramatic phenotypic changes in microtubules when treated with microtubule-stabilizing agents, such as taxol, because ovulated oocytes have many MTOCs [16]. In contrast, when treated with microtubule-destabilizing agents, such as vinblastine, their spindles rapidly disappear [17, 18].

In this study, we developed an oocyte-based screening system for anti-microtubule agents. Mouse ovulated oocytes were cultured with test samples for 5 h, and then oocytes were stained with anti-tubulin antibody and DAPI. The phenotypic changes in microtubules and chromosomes in treated oocytes were analyzed using fluorescence microscopy.

In the oocyte-based screening system, the effective dose (ED50) of taxol for microtubule polymerization was ~5 nM, and the ED50 of vinblastine for microtubule depolymerization was ~2.5 nM. In both cases, the detection sensitivities, 0.5–10 µM for taxol and 1–3 µM for vinblastine, were very high compared with those of the standard in vitro method [13,19, 20, 21]. In addition, taxol-like and vinblastine-like compounds could be evaluated simultaneously in a single assay using this system.

Material and Methods

Collection and culture of oocytes

The mice used in this study were ICR females, 3–4 weeks of age. To obtain the ovulated oocytes, female mice were injected with 5 IU of pregnant mare’s serum gonadotrophin (PMSG) and, 48 h later, with 5 IU of human chorionic gonadotrophin (hCG). Ovulated oocytes were collected from the ampulla oviducts 14–16 h after the hCG injection. Cumulus-enclosed oocytes were isolated, and the cumulus cells were dispersed by incubation for 2–3 min in 0.1% hyaluronidase in culture medium (modified Whitten’s medium containing 0.4% bovine serum albumin) at 38.5°C in a humidified atmosphere of 5% CO2 and 95% air. The cumulus-free oocytes were collected and rinsed three times in culture medium prior to further incubation in a plastic culture dish (35 × 10 mm, NUNC) [22].

Chemicals and plant extracts

Paclitaxel (taxol) and vinblastine were purchased from Sigma Chemical Co (St. Louis, MO). Methanol extracts of Taxus cupidata and Anthriscus sylvestris were purchased from the plant extract bank of the Korean peninsula (Plant Diversity Research Center in Korea). The chemicals and extracts were dissolved in dimethylsulfoxide (DMSO) and the concentration of DMSO applied to cells did not exceed 0.5%, a concentration that would have had no effect on microtubule phenotypic changes in oocytes.

Immunostaining of oocytes

Oocytes were fixed with 1.8% paraformaldehyde in PBS (fixing solution) for 40 min at room temperature (RT) and then permeabilized with 1% Triton X-100 in PBS for 20 min. Oocytes were then washed with 0.1% Tween 20 in PBS (washing buffer) for 20 min and incubated with PBS containing 3% BSA and 0.1% Tween 20 (blocking solution) for 1 h at RT. The oocytes were incubated with anti-tubulin antibody (YL1/2, Accurate Chemical) at a 1:100 dilution and then with fluorescein isothiocyanate-conjugated secondary antibody (Sigma Chemical Co., St. Louis, MO) at a 1:50 dilution. For both the primary and secondary antibodies, the incubation was performed for 1 h at RT. Oocytes were co-stained with 4,6-diamidinophenylindole (DAPI) to visualize DNA (Sigma). Immunostained oocytes were examined using a Zeiss fluorescence microscope (Thornwood, NJ).

Results

Phenotypic changes in oocytes treated with taxol

Ovulated oocytes have numerous MTOCs in their cytoplasmal, and some MTOCs form the spindle poles. Kinetochores on the chromosomes of oocytes can also promote microtubule growth.
To address the phenotypic changes induced by taxol, cumulus-free oocytes were cultured with taxol for 5 h and then stained with anti-tubulin YL1/2 antibody and DAPI (Fig. 1). Oocytes cultured with 50 nM taxol for 5 h showed spindle bundles and many astral-microtubules and dispersed chromosomes (Figs. 1c and c’). Some astral-microtubules appeared to elongate from the dispersed chromosomes. However, at low taxol concentrations of <10 nM, oocytes had fewer astral microtubules without dispersed chromosomes and no spindle bundles (Figs. 1b and b’). In this study, microtubule polymerization activity was evaluated based on the number of oocytes that displayed either spindle bundles or only astral-microtubules. Next, to investigate the sensitivity of the phenotypic changes produced by taxol, oocytes were cultured with specific concentrations of taxol for 5 h and immunoassayed with anti-tubulin antibody and DAPI. As shown in Table 1, taxol-induced phenotypic changes were observed in 50% of the oocytes treated with 5 nM taxol (ED50).

**Phenotypic changes in oocytes treated with vinblastine**

Mouse ovulated oocytes are arrested at metaphase in the second meiosis. In these oocytes, the structures of the metaphase spindles and chromosomes are well-defined. To investigate the sensitivity of the phenotypic changes produced by vinblastine, oocytes were cultured with specific concentrations of vinblastine for 5 h and immunoassayed with anti-tubulin antibody and DAPI. As shown in Table 2, vinblastine-induced phenotypic changes were observed in 50% of the oocytes treated with 20 nM vinblastine (ED50).

**Table 1. Effect of taxol (5 h incubation) on phenotypic changes of metaphase II arrested oocytes**

<table>
<thead>
<tr>
<th>Taxol (nM)</th>
<th>Total number of oocytes analyzed</th>
<th>Average number of oocytes with microtubule asters (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>4 ± 0.6 (4)</td>
</tr>
<tr>
<td>2.5</td>
<td>100</td>
<td>24 ± 4.5 (24)</td>
</tr>
<tr>
<td>5.0</td>
<td>100</td>
<td>52 ± 4.0 (52)</td>
</tr>
<tr>
<td>10</td>
<td>100</td>
<td>100 (100)</td>
</tr>
</tbody>
</table>

Data are the mean ± SEM of three experiments.

**Table 2. Effect of vinblastine (5 h incubation) on phenotypic changes of metaphase II arrested oocytes**

<table>
<thead>
<tr>
<th>Vinblastine (nM)</th>
<th>Total number of oocytes analyzed</th>
<th>Average number of oocytes without metaphase spindle (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>124</td>
<td>0 (0)</td>
</tr>
<tr>
<td>2.5</td>
<td>100</td>
<td>49 ± 3.5 (49)</td>
</tr>
<tr>
<td>5.0</td>
<td>100</td>
<td>89 ± 5.0 (89)</td>
</tr>
<tr>
<td>10</td>
<td>100</td>
<td>100 (100)</td>
</tr>
</tbody>
</table>

Data are the mean ± SEM of three experiments.
metaphase-aligned chromosomes were observed with tubulin and DNA staining. After oocytes had been cultured with vinblastine for 5 h, their spindle structures disappeared and (or) their chromosomes were dispersed (Figs. 1d and d’, e and e’). To investigate the sensitivity of these phenotypic changes produced by vinblastine, oocytes were cultured with specified concentrations of vinblastine for 5 h and stained with tubulin and DAPI. Vinblastine-induced phenotypic changes were observed in 50% of the oocytes treated with 2.5 nM vinblastine (ED50) (Table 2).

**Table 3. Effect of methanol extract of Taxus cupidata**

<table>
<thead>
<tr>
<th>Taxus cupidata (µg/ml)</th>
<th>Total number of oocytes analyzed</th>
<th>Average number of oocytes with microtubule asters (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>68</td>
<td>2 ± 0.6 (3)</td>
</tr>
<tr>
<td>5</td>
<td>65</td>
<td>11 ± 2.5 (17)</td>
</tr>
<tr>
<td>10</td>
<td>66</td>
<td>27 ± 4.4 (41)</td>
</tr>
<tr>
<td>20</td>
<td>68</td>
<td>67 ± 3.5 (99)</td>
</tr>
</tbody>
</table>

Data are the mean ± SEM of three experiments.

**Table 4. Effect of methanol extract of Anthriscus sylvestris**

<table>
<thead>
<tr>
<th>Anthriscus sylvestris (µg/ml)</th>
<th>Total number of oocytes analyzed</th>
<th>Average number of oocytes without metaphase spindle (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>72</td>
<td>0 (0)</td>
</tr>
<tr>
<td>1</td>
<td>70</td>
<td>3 ± 2.5 (4)</td>
</tr>
<tr>
<td>5</td>
<td>73</td>
<td>70 ± 2.0 (96)</td>
</tr>
<tr>
<td>10</td>
<td>70</td>
<td>69 ± 1.5 (99)</td>
</tr>
</tbody>
</table>

Data are the mean ± SEM of three experiments.

*Screening of methanol extracts from Taxus cupidata and Anthriscus sylvestris*

Taxus cupidata (Family: taxaceae) [23] is a source of taxol and Anthriscus sylvestris (family: Umbeliferae) contains a deoxypodophyllotoxin, which is known as a microtubule-depolymerizing agent [24]. Their methanol extracts were tested using our oocyte-based screening system. Metaphase arrested oocytes were cultured with various concentrations of the two different methanol extracts. The methanol extracts from Taxus cupidata showed microtubule-stabilizing activity with the number of microtubule asters in oocytes proportional to the extract concentration. Their ED50 was between 10–20 µg/ml concentrations in our oocyte-based screening system (Table 3). Also the extracts from Anthriscus sylvestris showed microtubule-destabilizing activity, disappearance of metaphase spindles in oocytes, and their ED50 was between 1–5 µg/ml concentration (Table 4, Fig. 2).
Discussion

A reverse chemical genetics system has generally been used to identify specific biological modulators or drug candidates [25]. This method in combination with a high-throughput system provides a very powerful approach to finding a compound against a known target using a chemical or natural products library [12, 13]. However, it is difficult to find novel compounds or new targets related to a specific phenotype because the application of this system is limited to known targets. On the other hand, a phenotype-based screening system, which is one of forward chemical genetics, is capable of identifying new compounds against known and unknown targets, although it requires more time and expense [26].

In this study, we demonstrated the phenotypic changes, i.e., large spindle bundles and microtubule asters that occur in oocytes cultured with taxol. Maro et al. [16] have also shown such changes, but at a relatively high concentration of taxol (0.4 µM). In our system, similar phenotypic changes were observed at a low taxol concentration (0.005 µM). The difference in sensitivities may be due to the difference in incubation times (5 h vs. 1 h). However, incubation times exceeding 5 h are not suitable for phenotypic analyses because the frequency of abnormal phenotypes, such as oocyte fragmentation, increases (data not shown).

We used immature mice to ensure freshly ovulated oocytes, ruling out the possibility of including post-ovulated oocytes. Freshly ovulated oocytes were collected by curetting the dilated ampullae of oviducts at 14 to 16 h. We selected the relatively small oocytes because frequently these oocytes already have microtubule asters. The average number of oocytes collected was 20 per mouse. Based on our experiments, we suggest that 7 to 10 oocytes are sufficient for one assay due to the good reproducibility of the assay.

To test our system for screening of plant extracts, we tried methanol extract from Taxus cupidata which contains taxol and Anthriscus sylvestris which contains deoxypodophyllotoxin [23, 24]. We could confirm the activities of both taxol and deoxypodophyllotoxin in our oocyte-based screening system which exhibited concentration dependency.

Additional biological activities can also be detected with this system. Metaphase-arrested oocytes can be activated by fertilization to re-start their cell cycles. Several chemicals are known to activate oocytes artificially. Protein synthesis inhibitors (puromycin, cyclohexamide) [27], PKC-activators (PMA) [28, 29], and CDK1-inhibitors (roscovitine) [30, 31] can activate metaphase-arrested oocytes. In this study, we also observed the phenotypic changes of activated oocytes; treatment with PMA or roscovitine induced chromosome decondensation and nuclear membrane formation (data not shown).

We have developed a novel oocyte-based screening system to analyze the activity of anti-microtubule agents. This system has a high sensitivity for taxol- and vinblastine-like effects as compared with the standard in vitro assay method. At least two biological activities can be evaluated simultaneously in a single assay. It may also be possible to identify new targets or chemicals using our system.

Acknowledgment

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References


